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IRVING H. LEOPOLD

Friedenwald Memorial

PROCEEDINGS

of the

Association for Research in Ophthalmology, Inc.

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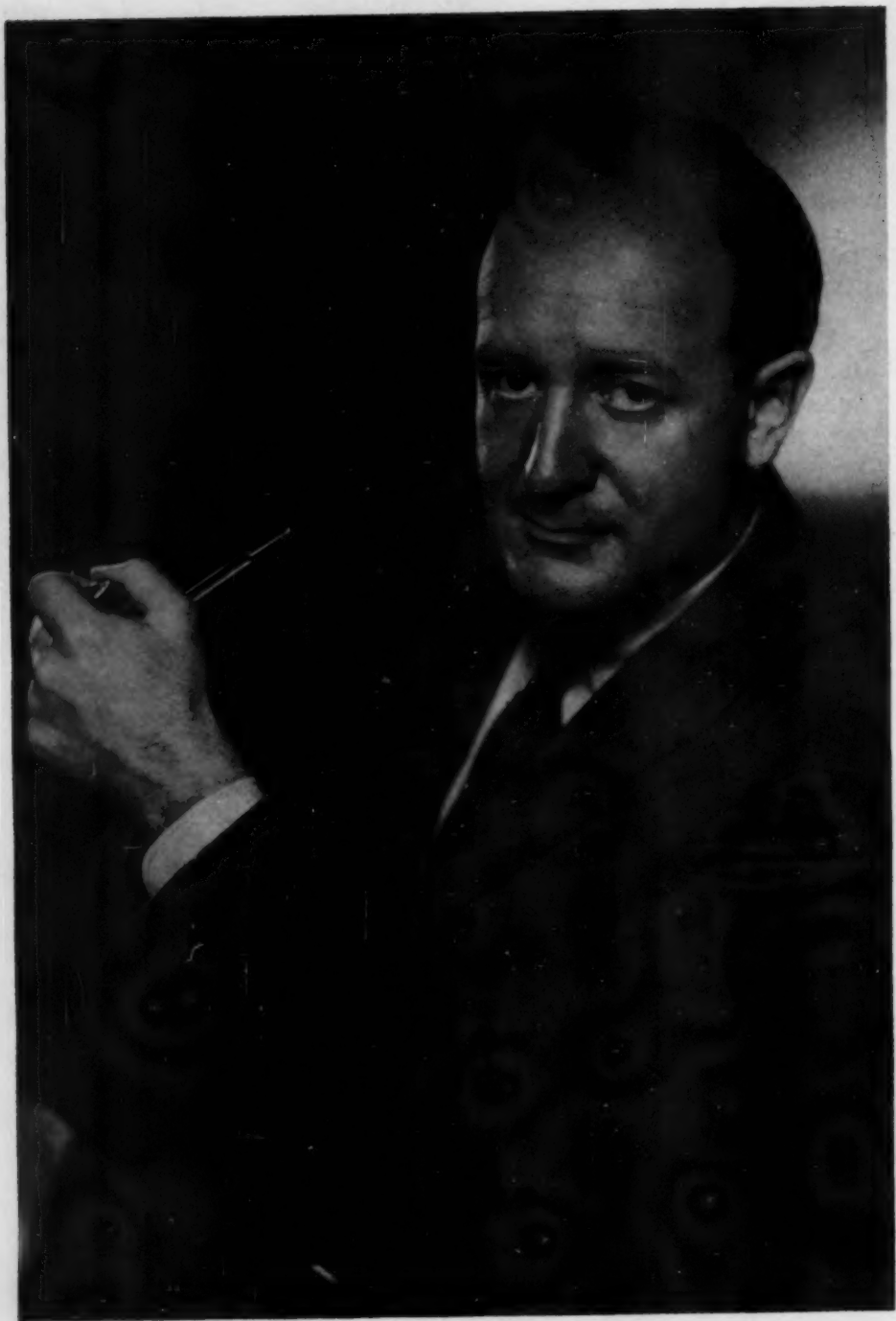
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IRVING H. LEOPOLD

COMMENTS ON ACCEPTANCE OF THE FRIEDENWALD AWARD

IRVING H. LEOPOLD, M.D.

Philadelphia, Pennsylvania

After recovering from the first pleasant warm shock of awareness that I had been singled out for a special award, a period of wonderment set in which was later replaced by a time of reflection. A doctor can study a disease all his life but not approach a real understanding of it until he himself has contracted it. I do not suggest a comparison between having a disease and receiving a coveted award, but there is a similarity in the strength of the stimulus provided for greater insight into meaning.

I can recall the first gold star placed on my music sheets by my piano teacher for meritorious performance of the scales. As I look back to that moment, I realize that the joy of being recognized was followed by a period of intense and persistent devotion to practice—even to the point of missing a few afternoons of football. I had confidence that if I practiced I would be able to perform satisfactorily, at least well enough for another gold star. My horizon may have been somewhat limited (really, it is difficult to remember) but I doubt that the music itself was nearly as important as the gold star. That I did not become a pianist of note I must attribute to my complete lack of musical talent and not to lack of encouragement or recognition.

Today in America there are probably as many awards given as there are areas of human endeavor and there are many reasons, other than merit, for conferring them. For example, there are sports awards for every kind and degree of athletic prowess and they are made as often to publicize the sport as to encourage the athlete. It is a reasonable assumption that a quarter-miler will run around a track as fast as his legs and lungs allow, and a silver cup at the finish line cannot make him go faster. The countless awards of the Miss America type yearly bestowed on thousands of lovely ladies cannot

make them any lovelier. The innumerable prizes given to theatrical and television performers rarely carry them on to greater acting achievements but frequently make them self conscious and fearful of being unable to repeat their performances. The various personalities chosen as subjects for the cover of *Time* magazine help sell the magazine but frequently condemn the poor subjects to a run of unaccountable bad luck. And it is the case with most awards that, given a hundred possible recipients, there will generally be one happy winner and 99 discouraged losers. The bestower will have made one friend and 99 enemies.

An award in medicine is something entirely apart and completely unique. It is something no doctor ever dreams of receiving, something he never expects. Actually it is a source of embarrassment, first because no doctor—especially a researcher—ever works alone and he feels guilty receiving a recognition which is equally merited by his many coworkers. Secondly, he knows how much more must still be done and so he feels undeserving. Because awards for what we do as doctors are not within our realm of thinking or expectation, we have no winners or losers, friends or enemies, because of them. Every advance in medicine is an encouragement to every physician, and it is this encouragement that makes recognition so meaningful.

Every indication of confidence is a spur to harder work. The young research worker, for instance, is sparked by each acceptance of a proposed research application given by a study section, a philanthropic group, or a research committee. The funds supplied are not more important than the confidence of which they are evidence. Increase in academic rank with the accompanying increase in responsibility is another bolsterer, another proof that the scientist is not like the

oyster which is cast away as soon as it produces the pearl. And nothing is as important in this area as the attitude of the man under whose direction a young doctor trains. In my case, Dr. Francis Adler's belief in me and my efforts was, and still is, an ever present source of encouragement.

Perhaps more than any other area of endeavor, research medicine is routinely disheartening and an award such as this restores confidence to all of us who are en-

gaged in it. "Our chief want in life," said Emerson, "is someone who will make us do what we can." As the gold star on my music scales pushed me to my strongest effort, so this award will "make me do what I can." I regard the Friedenwald Medal not as a recognition of past performance but as a calculated guess that, with this kind of encouragement and confidence, I will do the best job I possibly can in the specialty of ophthalmology.

BIOGRAPHY OF IRVING H. LEOPOLD, M.D.

Irving Henry Leopold was born in Philadelphia on April 19, 1915. Following education in the Philadelphia school system, he entered Pennsylvania State College in 1930. A brief foray with the football team resulted in a broken nose and collar bone, so he retired to more academic pursuits, which led to Phi Beta Kappa and the Evan Pugh medal for the highest honor in the college.

Early in his medical training at the University of Pennsylvania, Irving became interested in physiology, and worked with Bazzett and McCouch. This was the beginning of his prolific writing and of his special interest in biochemistry and physiology. He also found time while in medical school to marry his attractive wife Eunice (nee Robinson).

I first met Irving when he was interning at the University of Pennsylvania Hospital in 1938. It was quite obvious that he was destined for a position of eminence in whatever field he entered. In 1939 he started his career in ophthalmology in the Graduate School of the University, and joined the Department of Ophthalmology under Francis Heed Adler. During and following his residency, he worked as a research investigator in Chemical Warfare for the Office of Scientific Research and Development and the Committee on Medical Research of the National Defense Research Council, until 1945.

In 1949 he started the Research Depart-

ment of the Wills Eye Hospital and the phenomenal advance of this institution was recorded in this Journal in January of 1960. He is still the active director of the organization. Research activities did not preclude clinical practice and many hospitals sought, and obtained, his services. In 1952 he was appointed an Attending Surgeon at Wills Eye Hospital and has maintained an active service there.

His eminence as a teacher was recognized by his appointment as Professor of Ophthalmology and Chairman of the Department of Ophthalmology in the Graduate School of Medicine of the University of Pennsylvania in 1955.

Despite this heavy load, Irving is a consultant to more than a score of national organizations; is editor of one journal, and on the editorial boards of two others. He is in constant demand as a speaker and guest lecturer. There are few other people who can listen to a complicated technical presentation and then, without preparation, discuss it in terms less technical, and frequently more lucid, than the original presentation! Anyone who has heard Irving speak will admit that he has as much facility with the spoken word as with his writing.

His interests and accomplishments are not limited to the medical profession, however. He has more than a speaking knowledge of contemporary art and music; and he is an

avid sports fan, who can tell you what team won in 1936 or 1956, and by what score! An evening out with Irving is a real experience! He knows all the quaint eating places and the specialty of the house in each; and he can lead you to the spot where the latest combo is playing, or where the favorite blues singer is featuring her most famous platter.

His favorite sport is golf, though this suffers somewhat because of his peripatetic

activities.

The secret of Irving's success in his many interests may be his ability to give his undivided attention to each—whether it be his family, his friends, his research, or his books. His life is one of unselfishness. He gives of himself to his family, his patients, his research associates, and the many others who constantly call upon him for help and advice.

P. Robb McDonald, M.D.

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OCULAR CHOLINESTERASE AND CHOLINESTERASE INHIBITORS

THE FRIEDENWALD MEMORIAL LECTURE

IRVING H. LEOPOLD, M.D., D.Sc. (MED.)*

Philadelphia, Pennsylvania

Frazer observed the miotic action of the alkaloid derived from the dried ripe seed of the calabar plant, *physostigmina venosum*, in 1863. The miosis came on promptly, reached its maxima in one-half hour following installations of $\frac{1}{4}$ to $\frac{1}{2}$ percent solution. Such an induced miosis persisted for twelve to thirty-six hours. Also in 1863 Argyl-Robertson demonstrated that locally instilled physostigmine could overcome the pupillary effects of atropine and by 1875 Laquer had successfully employed physostigmine locally in the therapy of glaucoma.

In 1905 Anderson studied the pharmacology of physostigmine and demonstrated that physostigmine can constrict the pupil in normal cats but fails to do so after the ciliary ganglion has been removed and time allowed for degeneration of the post-ganglionic fibers. It was not until 1918 that Hunt first described the potentiating action of physostigmine on the action of acetylcholine. Stedman elucidated the chemical structure of physostigmine in 1925. In the course of the studies of Stedman and his associates on the relationship of miosis to the chemical basis of these compounds, Aeschlimann and Reinert synthesized prostigmine or neostigmine. Most of the classical work of Stedman and collaborators and Aeschlimann and Reinert on the relationships between chemical structure and pharmacological activity of the homologues of physostigmine and related compounds was conducted before it was realized that these drugs were potent inhibitors of choline esterase.

Drug action is usually thought to be due either to interference with enzyme activity or to a combination of the drug with specific receptors of an effector organ. Anticholin-

esterase agents constitute one of the few classes of drugs for which the mechanism of action has been defined in terms of inhibition of the specific enzyme. Prior to World War II this enzyme, cholinesterase, was of purely academic interest. The advent of the organophosphorus anticholinesterase agents brought new aspects to the subject and cholinesterase determinations have become of practical importance for a number of people such as workers in industrial health, crop protection, chemistry of insecticides and chemical warfare agents. The irreversible nature of some of the alkylphosphate type of anticholinesterases has made them useful tools in various fields of biology, enzymology, protein chemistry, physiology, pharmacology and toxicology. The literature has become huge, and investigators all over the world are using these compounds for a variety of practical and theoretical problems. An enormous amount of data of all kinds about the organophosphorus cholinesterase inhibitors has accumulated in the past 15 years, summed up in many reviews. These have been concerned mostly with their chemistry, biochemistry and insecticidal activity. A most extensive paper on pharmacology of anticholinesterase agents was written ten years ago by Koelle and Gilman and more recently by Holmstedt in 1959. The rapid growth of the field since 1949 has prompted many other reviews devoted to specific topics such as intoxication with insecticides.

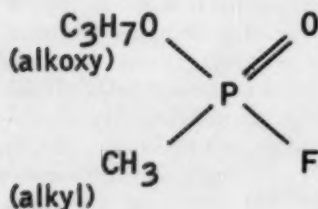
Although organic phosphorus compounds such as tetraethylpyrophosphate (TEPP) have been known since the middle of the last century (DeClermont, 1854), it was not until immediately before World War II that vigorous interest in their chemistry occurred, largely due to Schrader. His pioneer

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work began in the middle thirties at the FarbenFabrikan Bayer in Germany. These organophosphorus compounds were used by Schrader in the 1930's for the development of insecticides. Some of the compounds are found to be volatile and extremely toxic, especially for the nervous system. The German Army took hold of them and developed the famous nerve gasses as potential chemical warfare agents, the most toxic compounds ever developed. Some of them are the most potent enzyme inhibitors known.

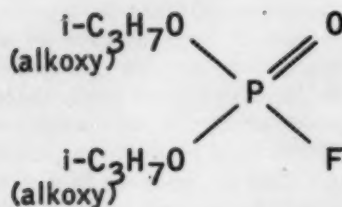
Among the important compounds synthesized by Schrader are diethyl paranitro phenylphosphate or paraoxon, diethyl paranitro phenyl thiophosphate or parathion, diethyl-2-ethylthioethyl thiophosphate (Sys-

tox), deimethylamido ethoxyphosphoryl cyanide or Tabun, and isopropoxymethylphosphorylfluoride or Saran. The last two compounds, because of their high toxicity, are regarded as potential warfare agents and were kept secret for several years. With the exception of their formulas which were published by Valade and Salle and Bonnaud, no information about these compounds appeared in the literature until the early 1950's. In England an intensive research program in the field of organophosphorus compounds was pursued during World War II largely under the direction of McCombie and Saunders. Their work resulted in a series of di-alk-oxyphosphoryl fluorides. One of the most interesting of these compounds



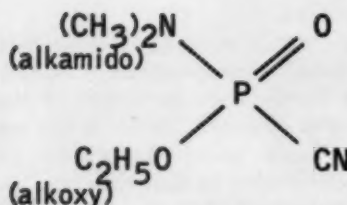
SARIN

isopropyl methyl phosphonofluoridate



D.F.P.

diisopropyl phosphorfluoridate



TaBun

ethyl-N-demethyl phosphoramido cyanidate

Chart I (Leopold) Chemical formulas of potential warfare agents.

BASIC FORMULA FOR ANTICHOLINESTERASES

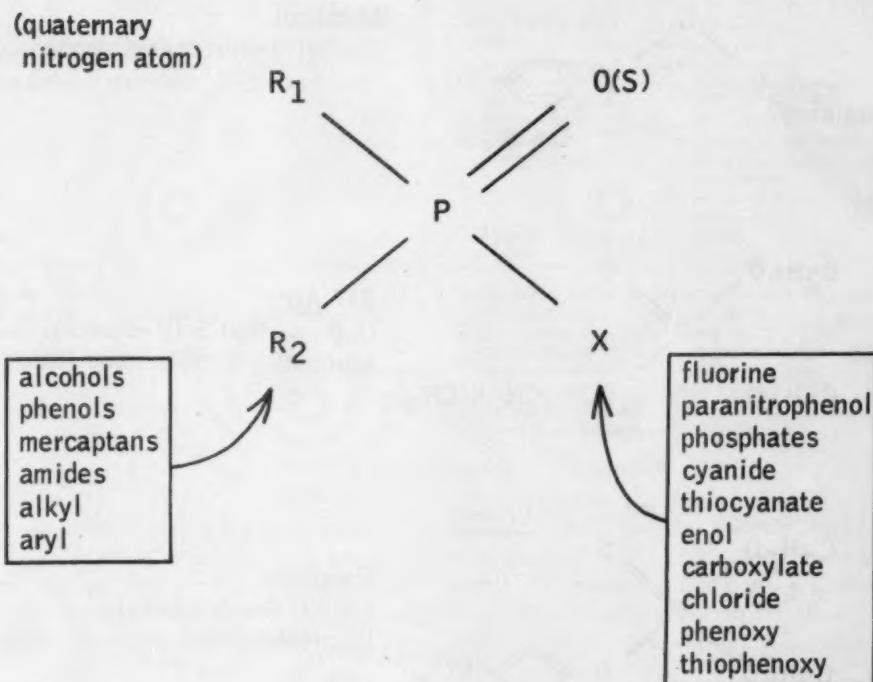


Chart II (Leopold)

from the ophthalmological standpoint is diisopropyl-fluorophosphate (See Chart I).

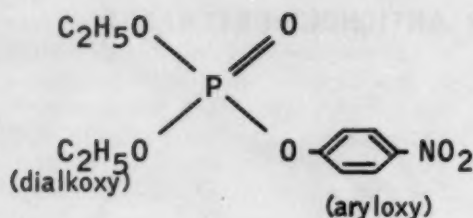
GENERAL FORMULA OF ORGANOPHOSPHORUS ANTICHOLINESTERASES

The general formula which can be applied to cholinesterase inhibitors of the organophosphorus group was described in 1937 by Schrader. This is the general formula for derivatives of phosphorus acid with insecticidal properties (See Chart II). It implies that biologically active compounds are obtained when in addition to oxygen or sulphur two similar or dissimilar substituents are bound to the phosphorus atom and an organic or inorganic acid residue is also present. Examples of some of these compounds are listed in the accompanying charts III and IV.

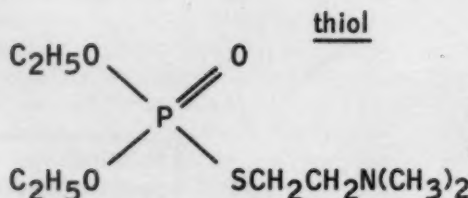
During the past twenty years a great number of compounds have been tested for their ability to inhibit cholinesterase. Augustinsson in 1948 reviewed almost 900 publications devoted to inhibitors of the enzyme and their pharmacologic effects.

Enzymes: Enzymes which hydrolyze esters are known as esterases. They may be divided in three groups: sulfatases, phosphatases, and carboxyl esterases. The last group include ali-esterases. These are enzymes which hydrolyze esters of acids with aliphatic chains containing fewer than twelve carbon atoms. The carboxylases also include lipases, cholinesterases, acetyl esterases, cholesterol esterases, lecithinases, and other enzymes such as atropin-esterase.

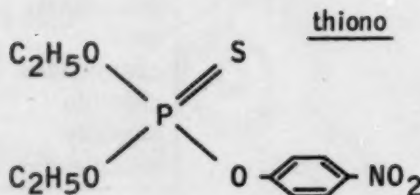
The fundamental work of Alles and Hawes (1940) demonstrated that there are



Paraoxon
Mintacol
diethyl 4-nitrophenyl phosphate



217 AO
O,O, diethyl S (2-dimethyl
amino ethyl) phosphorothioate



Parathion
diethyl 4-nitrophenyl
thionophosphate

Chart III (Leopold)

two types of cholinesterase, specific or true cholinesterase or, in the nomenclature of Augustinsson and Nachmansson (1949), acetylcholinesterase and nonspecific cholinesterase, pseudocholinesterase or simply cholinesterase. These enzymes differ by the rate of hydrolysis of substrates; it decreases for acetylcholinesterase and increases for cholinesterase with increasing length of the acyl group from acetyl to butyryl. Acetylcholinesterase is inhibited by an excess of acetyl or propionyl choline while an excess of substrate even of butyryl does not inhibit nonspecific cholinesterase. Biochemists make use of substrates that are hydrolyzed by one type of cholinesterase but not by another, e.g. acetyl-beta-methylcholine by acetylcholinesterase and benzoyl-choline by cholinesterase. There is not absolute specificity, for

acetylcholinesterase can split some acetyl esters and bases other than choline and the separation between the two types is not as clear as at first suggested. (See Table II).

The organophosphorus compounds are specific inhibitors of enzymes capable of splitting ester linkages. They inhibit all types of esterases, lipases, trypsin, chymotrypsin and thrombin. They belong to the so-called irreversible inhibitors, i.e. their action is not reversed by simple dialysis but only by chemical reaction. Not all esterases are inhibited. The phosphoryl phosphatases use the organophosphorus esters as substrates (See Table II).

The Localization of Cholinesterase: Until recently the cholinesterases in tissues have been studied manometrically using homogenates of tissue slices. The methods employed

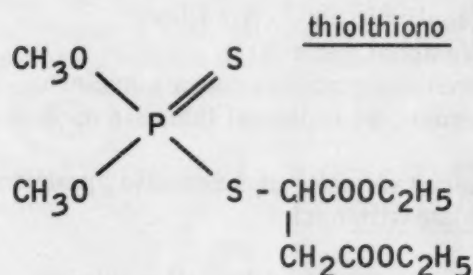
CHOLINESTERASES

	<u>Occurrence</u>	<u>Occurrence in Mammals</u>	<u>Substrate Specificity</u>	<u>Selective Inhibitors</u>
Aceto CHE			<u>Choline Esters</u>	<u>Some biquaternary ammonium bases particularly active.</u>
Acetyl CHE	Electric fishes	CNS grey substance	Splits choline esters or thiocholine esters in the following order:	e.g. <u>Reversible Inhibitors</u>
Specific CHE		Erythrocytes	Acetyl choline	WIN 8077
True CHE	Cobra toxin	Sympathetic ganglia	Propionyl choline	NU 1250
		Motor end plates	Butyryl choline	<u>Irreversible Inhibitors</u>
			Splits acetyl B-methylcholine	methyl-fluoro-phosphoryl
				homocholine
				methyl-ethoxy-phosphoryl
				thiocholine
				BC 48
Butyro CHE		Plasma	Splits choline esters or thiocholine esters in the following order:	<u>Organophosphorous compounds</u>
Butyryl CHE	Human plasma	Intestinal mucosa	Butyryl choline	Iso OMPA
Nonspecific CHE		Pancreas	Propionyl choline	DFP
Pseudo CHE	Dog pancreas	Liver	Acetyl choline	All phosphorous compounds of irreversible type
		CNS white substance	Splits benzyl choline but not acetyl B-methylcholine	<u>Reversible Type</u>
				Lipivane
				NU 683

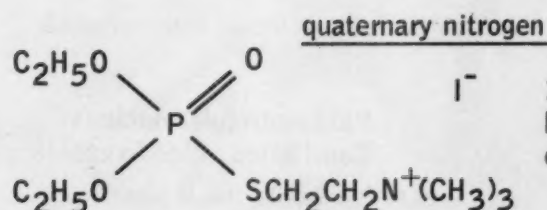
Table I (Leopold)

have been reviewed by Augustinsson in 1957. This type of study does not reveal in which cell or parts of cells the enzyme activity is located. Three techniques have been employed more recently in answer to this

problem. They include the histochemical techniques, cartesian diver technique and centrifugal fractionations of tissue homogenates. Histochemistry borrows most of its techniques from microchemistry, but only a



Malathion
O,O, dimethyl S,(1-2,
dicarbethoxy ethyl)
thiothionophosphate



I⁻ 217 MI
Phospholine
diethoxyphosphoryl -
thiocholine iodide

Chart IV (Leopold)

OTHER ENZYMES REACTING WITH ANTICHOLINESTERASES

Ali-esterases (Chymotrypsin) (Trypsin)	Occurrence in Mammals	Substrate Specificity	Substrate Specificity	Selective Inhibitors
	Liver Brain Plasma Tumors Pancreas	Does not split choline esters	Other esters Triacetin Tributyrin Ethylacetate	DFP TEPP Isoparaoxon
Phosphoryl phosphatases (hydrolyze phosphorous esters)	Plasma Adrenal gland Liver Kidney Spleen Heart Lung Brain Muscle Skin	Does not split choline esters, tributyrin	Organophosphorous compounds like: DFP TEPP TaBun Sarin Ethylacetate Phenylacetate	Hg ⁺ Cu ⁺⁺ No inhibition by organophosphorous CHEI

Table II (Leopold)

HISTOCHEMICAL LOCALIZATION OF CHOLINESTERASE
BRAIN

AcChE (Intense Staining) :

Motor neurons of cranial motor nuclei
 Neurons giving rise to preganglionic autonomic fibers
 (those of Edinger-Westphal nucleus)
 Neurons of the reticular formation; gracilis, cuneate nuclei
 Certain tertiary afferent neurons, as in lateral thalamic nucleus

Hypothalamus: Confined to certain cells of supraoptic, paraventricular and suprachiasmatic nuclei

Enzymatic staining: Most concentrated in the cell membrane;
 dendritic and axonal prolongations

BuChE:

White fiber tracts
 Glia cells
 Supraoptic nucleus

Paraventricular nucleus
 Capillaries, blood vessels

Table III (Leopold)

HISTOCHEMICAL LOCALIZATION OF CHOLINESTERASE GANGLIA

AcChE :

Cholinergic neurons

Adrenergic neurons: variable amounts; some cells no stain

Sensory neurons: " "

BuChE :

Glial cells of ganglia

Cells of Auerbach's plexus

Table IV (Leopold)

few of these can be adapted safely to microscopic preparations. Quantitative measurements are generally impossible in histochemistry or subject to such errors that the logical step is to complement histochemical research with microchemical investigation as Zajicek did for acetylcholinesterase and blood platelets in 1957. Histochemistry, in spite of its quantitative and even qualitative limitations and the unreliability of some of the techniques, holds a central position at

the convergence of morphological, physiological and biochemical research.

Up until the present moment, there has been no histochemical technique for detection of acetylcholine. Acetylcholine synthesis could be studied by the localization of cholineacetylase. Biochemical surveys of its distribution have given favorable results in the hands of Feldberg and Vogt in 1948, Hebb in 1955, Hebb and Silver in 1956, although they do not take into account the rate-limiting ef-

HISTOCHEMICAL LOCALIZATION OF CHOLINESTERASE SPINAL CORD

AcChE :

Anterior and lateral horn: large motor neurons

BuChE :

Capillaries and blood vessels

Table V (Leopold)

HISTOCHEMICAL LOCALIZATION OF CHOLINESTERASE STRIATE MUSCLE

AcChE :

Musculotendinous junctions (stretch receptors?)

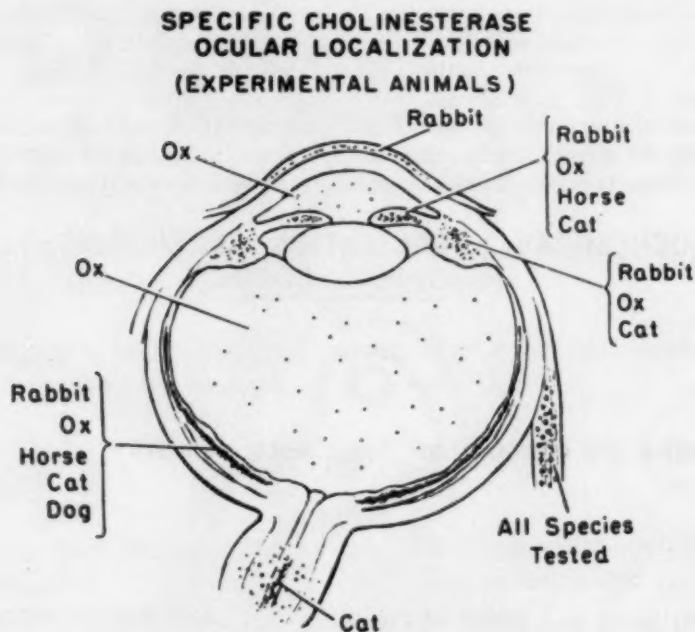
AcChE and BuChE :

End plates - amounts exceed that in rest of striate muscle

BuChE :

Muscle spindles

Table VI (Leopold)



NON-SPECIFIC CHOLINESTERASE OCULAR LOCALIZATION.

INCREASED PERMEABILITY
-VASCULAR DILATATION-

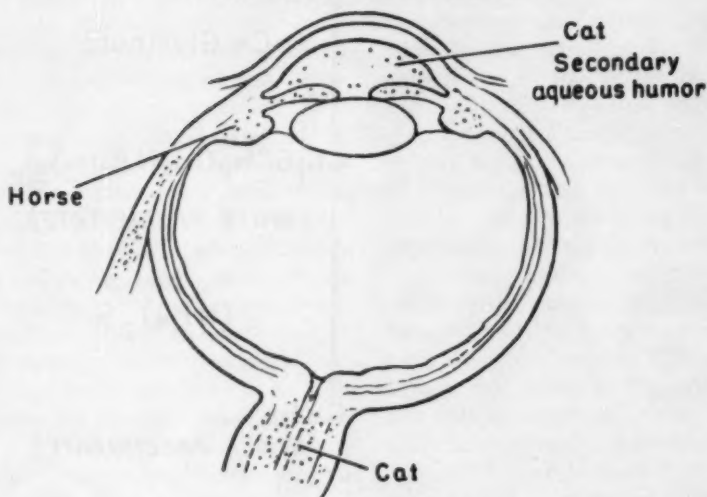


Diagram II (Leopold)

fect of acetyl co-enzyme A concentrations (Smallman, 1958). Such a histochemical method, however, does not furnish indications of the acetylcholine release sites since the enzyme appears to be produced in the neuronal cell bodies (Feldberg and Vogt, 1947; Hebb and Waites, 1956). On the contrary, the speed of acetylcholine hydrolysis by specific cholinesterase excludes the existence of an important gap between the structures releasing acetylcholine and those containing the cholinesterase enzyme so that this has been the subject of considerable study. Actually it has inspired more than 4000 articles which have been reviewed by many including Augustinsson in 1948, Hebb in 1957 and Paton in 1958.

Histochemical Techniques for CHE: Two groups of techniques are now available. The first one makes use of specific substrates and proceeds from the method of Koelle and Friedenwald in 1949 (Chart V). The other group employs nonspecific substrates and

then differentiates between the various carboxylesterases with the help of specific activators and inhibitors (Barnett & Seligman, 1951). Each method has its drawbacks. Sensitivity must be sacrificed to precision in localization. Long practice is necessary to evolve the technique most appropriate for the material under study and ends to be obtained. It is essential that biochemically-controlled experiments be run on exactly the same solutions as those employed for histochemical studies. Shifts in Ph will alter the concentration of inhibitor necessary to block one type of enzyme selectively (Holmstedt, 1957).

Some authors have used fixed material instead of frozen sections for these histochemical studies, and this has usually been formalin fixation. Very few biochemical controls exist for experiments with formalin. Taxi studied the effect of formalin on cholinesterase manometrically. He employed preparations from various sources and various de-

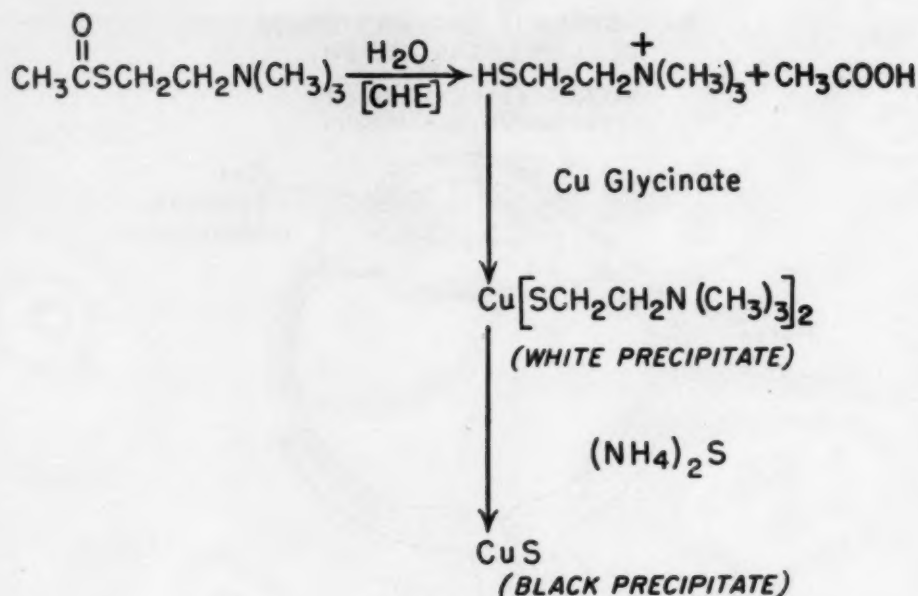


Chart V (Leopold)

degrees of purity, and found that those preparations of high purity were more susceptible in the inactivation of formalin than others. Electric organ cholinesterase was completely inactivated by 10 per cent formalin in 78 minutes whereas cholinesterase of ox caudate nucleus retained 10 to 20 per cent of its original activity at the end of this time. Butyryl cholinesterase from various sources, e.g. from serum and from pancreas, differed in susceptibility.

Localization of Cholinesterase in Body: The accompanying tables nos. III, IV, V and VI list the sites at which cholinesterase has been localized by histochemical technique and wherever possible confirmed by manometric analysis.

There is widespread distribution of acetylcholine in striate muscle, and considerable discussion as to the exact situation in which this enzyme has been found. However, there appears to be overwhelming evidence that the content of the enzyme in the motor end plates is far above that of any other structure in striated muscle (Holmstedt 1959).

Localization of Cholinesterase in Ocular Tissues: There have been a number of papers written on experimental attempts to localize cholinesterase in ocular tissues of experimental animals. Unfortunately, most of the early investigations paid little attention to the properties of this enzyme such as its stability, the optimum Ph for determination, the importance of enzyme and substrate concentration and of temperature range. Most of them employed biological methods which are not accurate. The subject has been reviewed ably by DeRoeth (1950). DeRoeth used the chemical method developed by Hestrin (1949). The values he obtained were in very close agreement with those reported earlier by Nachmansohn (1940) but much higher than those of Bruckner (1943), Herrman and Friedenwald (1942). The most likely reason for the lower values of the earlier authors was the excessive concentration of substrate which had been employed by these investigators. The homogenation process employed in the study may also be a factor.

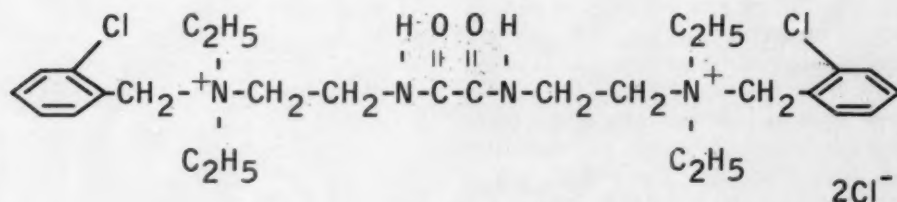
By means of the Hestrin test, DeRoethth concluded that cholinesterase enzyme activity was highest in the muscle and nerve-containing tissue such as the iris, ciliary body, and retina (Diagrams I and II). The aqueous humor and vitreous contain only traces of this enzyme whereas the retinas of the various animals studied, i.e. rat, cat, kitten, dog, pigeon, rooster, pig, ox and horse showed approximately the same amount of enzyme activity. The irides and ciliary bodies varied considerably in cholinesterase concentration from one species to another. It appeared that the cholinesterase activity in the iris and ciliary body in these different species was related to the amount of muscle present in these tissues. DeRoethth also attempted to demonstrate the presence of specific and nonspecific cholinesterase in these ocular tissues. He was fully aware that there might be both types present, but there was a good chance of overlooking the one with the lower concentration. He was able to demonstrate that the iris, ciliary body and serum of the rabbit; the iris, ciliary body, aqueous and vitreous of the ox; the iris of the horse; the iris and ciliary body of the cat; the retina of the dog, cat, rabbit, ox and horse all demonstrated an hydrolysis product typical

of the specific cholinesterase. Nonspecific cholinesterase was observed in the ciliary body of the horse; the serum and secondary aqueous of the cat. He was able to prove that, in the secondary aqueous of the cat, the cholinesterase was derived from the serum. Probably that which was present in the aqueous and vitreous of the ox eye was derived from the iris, ciliary body and retina (See Diagrams I and II).

From the pharmacologic and enzymological investigations, it has been suggested repeatedly that acetylcholinesterase is located both inside and outside the cell membrane (Schweitzer, Stedman and Wright) (Burgin and Chipman 1952) (Koelle and Steiner 1956). Such suggestions have been based upon comparisons of the effects of tertiary and quaternary ammonium bases with anticholinesterase activity. It is known that the quaternary compounds have less penetrability due to their lipid insolubility.

Koelle has recently used quaternary compounds, e.g. Mytelase, to protect the enzyme in vivo from attack of irreversible inhibitor, DFP, and then to stain histochemically the protected enzyme (See Chart VI). The principle of protecting the enzyme from the action of DFP with a reversible quaternary

WIN 8077



N, N' - Bis - (diethyl - 2 chloro benzyl - ammonium ethyl) oxamide dichloride

(Ambenonium)
(Myletase)

Chart VI (Leopold)

cholinesterase inhibitor was employed to determine the sites of inhibition of the enzyme by the latter compound. Histochemical sections of ganglia of cats which received only DFP were completely blank. The motor end plates were stained faintly. By contrast, the ciliary and stellate ganglia and motor end plates of cats which previously had received increasing doses of protective reversible inhibitors, ambenonium and derivatives, showed progressive staining. Staining was most marked at the borders of the neurons in the ciliary ganglion and in the intercellular plexes of the stellate ganglion. The cytoplasm of the cells in the ciliary ganglion which stained intensely in untreated controls remained blank. The cholinesterase within the cell was not protected by the ambenonium from inhibition by DFP and therefore this enzyme was not free to act and no stain developed in the cytoplasm.

There has been controversy concerning the existence of acetylcholinesterase in adrenergic neurons. Holmstedt and Sjöqvist in a detailed study, using a revised histochemical technique, counted the cells in 4 sympathetic ganglia of the cat and found great differences in the number of stained cells. These were divided arbitrarily into heavily-stained, faintly-stained and unstained cells. The number of cells stained at all was as follows: the superior cervical ganglion, 21.3 per cent; stellate ganglion, 14.9 per cent; superior mesentery ganglion, 6.8 per cent; coeliac ganglion, 4.8 per cent. This is in contrast to the findings of Koelle, but agreeing with those of Giacobini who used a microdiver technique. Whatever the explanation of these differences, the evident lack of pharmacologic and physiologic homogeneity of the cell populations of sympathetic ganglia should put us on guard against drawing too far-reaching conclusions regarding transmission from experiments on one ganglion.

Equipotent doses of phosphoryl compounds containing tertiary and quaternary nitrogen groups respectively give a very

different degree of inhibition of the subsequently homogenized sympathetic ganglion. It can, however, be shown that with the quaternary compounds the inactivation occurs predominantly in pre-ganglionic terminations and peripheries of ganglion cells whereas internal cytoplasmic acetylcholinesterase is relatively unaffected. This suggests that the latter is not immediately involved in the hydrolysis of acetylcholine during cholinergic transmission.

The concept of dual localization of the enzyme is important and must influence significantly any discussion of pharmacologic action in terms of cholinesterase activity. If these investigations can be confirmed and extended, they may explain many hitherto unexplained facts in the pharmacodynamics of cholinesterase inhibitors.

Electron Microscopic Localization of Cholinesterase: Experiments which combined histochemistry and electron microscopy have been performed (Barnett & Palade 1959). This technique studied the sites of enzymatic hydrolysis by the theolacetic acid technique in the presence of lead ions in diaphragmatic and cardiac muscle. It was found that in these striated muscles the electron opaque, final product of the histochemical reaction (PbS) was discretely deposited on the swelling of thick elemental filaments that occur at the M band.

Cholinesterase has also been demonstrated at the myoneural junction of intercostal muscle in electron microscopic preparations utilizing a diazo coupling technique (Lehrer & Ornstein 1959).

In Vivo Effects of Cholinesterase Inhibitors As Related to Biochemical Determinations: Although most research workers attribute the pharmacologic action of anticholinesterases solely to enzyme inhibition and their effects to accumulation of acetylcholine, relatively few papers have been devoted to this topic. Michaelis and his coworkers determined the acetylcholine content of one hemisphere of the rabbit brain after injection of DFP, the other half having been

taken out first as a control. Experiments proved that DFP caused a significant increase in acetylcholine content which persisted for at least a half hour. The injection of saline did not change the high values of acetylcholine one-half hour after DFP. Neither did atropine given before, although it brought about a decrease in the severity of clinical symptoms such as salivation, fasciculation and convulsions. In other experiments the anticholinesterases, TEPP, DFP and eserine in lethal doses all produced a significant increase in total acetylcholine in the brains of rats over that of rats killed by asphyxia, chloroform or strychnine (Stewart 1952).

Acetylcholine-like substances circulating in the blood have been demonstrated after intoxication with anticholinesterases. From the data which is available, there can be little doubt that organophosphorus compounds can produce increases in the concentration of acetylcholine or acetylcholine-like substances in the blood and tissues. Lethal doses accompanied by artificial respiration and huge doses of antidotes are necessary to achieve this, and it is likely on the basis of our present knowledge of transmission that the local release and accumulation of choline esters at strategic points is a more important factor in the pharmacodynamics of cholinesterase inhibition than the rise in the choline ester content of the circulating blood.

It is assumed that by preventing the rapid enzymatic inactivation of acetylcholine the anticholinesterase agents allow acetylcholine to exert its characteristic action in an intensified fashion and thus muscarinic actions of acetylcholine on smooth muscle, secretory glands, heart; nicotinic action on skeletal muscles and autonomic ganglion will be increased by the use of anticholinesterase agents.

After the cholinergic nerves to smooth muscles, gland cells, and ocular structures are resected and allowed to degenerate, acetylcholine is thought to be no longer liberated locally (See Table VII). Under such

circumstances, anticholinesterase agents fail to manifest their characteristic effects. This dependence of the action of anticholinesterase agent upon intact innervation was first clearly demonstrated by Anderson in 1905. He employed physostigmine and found that the pupil did not constrict in response to the drug when the ciliary ganglion was removed. The observations of Anderson were confirmed by Leopold and Comroe in 1945 and extended to neostigmine. In addition, the stimulating effect of eserine on normally-innervated smooth muscle is characterized by a long, latent period, by slow developing and in in vitro experiments, by its gradual disappearance when the drug is washed out. These features are strongly suggestive of an indirect mechanism of action. Unlike neostigmine, physostigmine exhibits no component of direct action on skeletal muscle. Intra-arterial physostigmine does not produce an immediate contractile response in voluntary muscles and the drug is without effect on chronically denervated mammalian skeletal muscle (Wescow and Riker 1951). It is thus concluded that physostigmine exerts its pharmacologic effects solely as a result of its anticholinesterase activity. Similar experiments carried out for di-isopropyl fluorophosphate allows the same conclusions to be drawn for this action of DFP (Leopold & Comroe) and for phospholine iodide (Leopold). Neostigmine, by virtue of the fact it has direct effect on skeletal muscles according to Wescow and Riker and others, may differ in its activities to this extent.

Role of Erythrocyte Cholinesterase Content: The relationship between erythrocyte cholinesterase and symptomatology depends to a large extent on the route and rate of administration. Unprotected men exposed to the vapor of DFP or sarin can suffer extreme discomfort without perceptible change in cholinesterase activity of red blood cells. Masked men exposed to skin contamination can lose up to 85 per cent of their cholinesterase activity of the red blood cells before beginning to indicate symptoms. Compara-

REACTION OF PUPIL before and after POST-GANGLIONIC PARASYMPATHETIC DENERVATION.		
DRUG -Local-	NORMAL EYE. DEGREE OF PUPIL CONSTRICTION.	CILIARY GANGLION EXCISED. POST-GANGLIONIC FIBERS DEGENERATED. DEGREE OF PUPIL CONSTRICTION.
1 % Pilocarpine	++	+++
20 % Mecholyl	++	+++
2.5% Mecholyl	0	++
10 % Acetylcholine	0	+
0.5% Physostigmine	++	0
0.1% Diisopropyl fluorophosphate	++	0
5 % Prostigmine	++	0
0.25% Phospholine	++	0

Table VII (Leopold)

tively large oral or intravenous doses produce symptoms with little inhibition of red cell cholinesterase whereas repeated small doses can produce a considerable fall in cholinesterase without symptoms (Holmstedt 1959). This can be demonstrated through analyses of serum cholinesterase, plasma and whole blood cholinesterase following repeated small instillations of anticholinesterase agents into the ocular cul-de-sac as demonstrated by Leopold, Krishna and Lehman in 1959.

Recovery of erythrocyte cholinesterase is slow. Regeneration of cholinesterase has been studied in humans and in experimental animals. The rate of regeneration of erythrocyte and brain cholinesterase activity in rats after exposure to sarin has been examined and treated statistically by Oberst and

Christensen (1956). From their data, it is estimated that 48 plus or minus 6 days were required for complete recovery of erythrocyte cholinesterase. It required about 140 plus or minus 31 days for the brain cholinesterase to recover completely. Approximately 50 to 80 per cent of total acetyl cholinesterase of nervous tissue or effective organs must be inactivated before pharmacologic actions are noted. More recent experiments indicate that, during repeated exposures to low concentrations of serum, brain cholinesterase inhibition is slight until erythrocyte cholinesterase activity falls below 30 per cent of normal and the toxic signs are not severe until marked brain cholinesterase depression has occurred below 10 per cent of normal.

It is evident then that patients who will be

receiving phospholine iodide or humorsol over a long period of time must be watched with the possibility that there may be a marked depression of erythrocyte and serum cholinesterase and symptoms arise due to this. From the data which is accumulated on patients already treated, this will probably be a very rare complication, but nevertheless it is a possibility that must be kept in mind (Leopold, Krishna & Lehmann 1959) (See Table VIII).

The Relationship of Serum and Red Cell Cholinesterase to Glaucoma: The exact etiology and pathogenesis of glaucoma still remains unknown. One of the cardinal features of this symptom and sign complex is the increase in intraocular pressure. Failure to control this increase in intraocular pres-

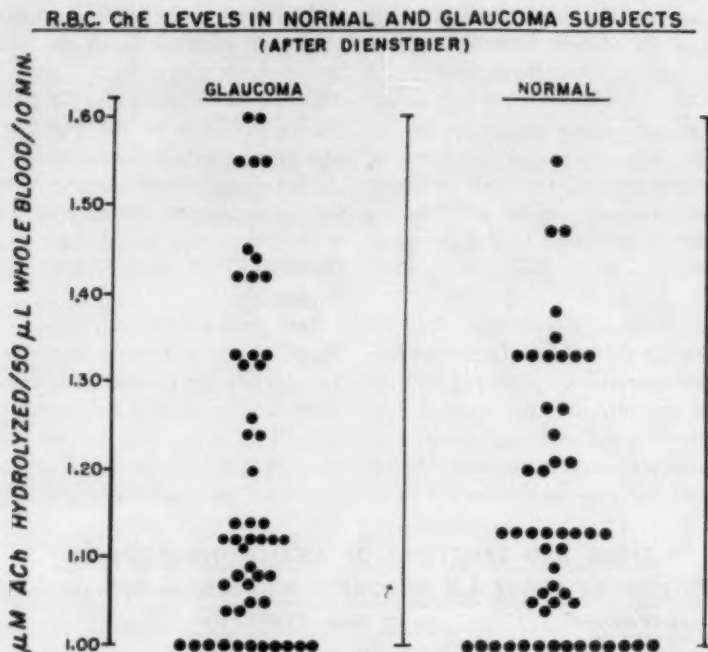
sure results in irreparable damage to the eyes and eventual blindness. Anticholinesterase agents have been found to be very effective in lowering this increase in intraocular pressure in the glaucomatous eyes and bring it within normal limits. Because of the beneficial effects exerted by the anticholinesterase agents, attempts have been made from time to time to associate glaucoma with disturbance of acetylcholine-cholinesterase-mechanism.

One such attempt has been to find out if there is any difference between the blood ChE levels of normal and glaucoma subjects. Rados failed to find a change in whole blood ChE levels in glaucoma subjects (Rados 1943) but these results are not surprising since the unchanged plasma would ob-

SIGNS AND SYMPTOMS OF ANTICHOLINESTERASE FOLLOWING SYSTEMIC ABSORPTION. (Grob & Harvey; Holmstedt)

SITE OF ACTION	SIGNS AND SYMPTOMS
BRONCHIAL TREE	Tightness in chest, with prolonged wheezing expiration suggestive of bronchoconstriction or increased secretion, dyspnea, slight pain in chest, increased bronch. secr., cough.
GASTRO-INTESTINAL	Anorexia, nausea, vomiting, abdominal cramps, epigastric & substernal tightness (?cardiospasm) with "heart-burn" and eructation, diarrhea, tenesmus, involuntary defecation.
HEART	Slight bradycardia.
SWEAT, SALIV. & LAC. GLANDS	Increased Sweating, Salivation and Lacrimation.
PUPILS	Slight miosis, occasionally unequal, later more marked miosis.
CILIARY BODY	Blurring of vision.
BLADDER	Frequency, involuntary micturition.
STRIATED MUSCLE (NICOT.-LIKE)	Easy fatigue, mild weakness, muscular twitching, cramps, fasciculations, generalized weakness, including muscles of respiration, with dyspnea and cyanosis.
SYMPATHETIC GANGLION	Pallor, occasional elevation of blood pressure.
CENTRAL NERVOUS SYSTEM	Giddiness; Jitteriness; restlessness; Insomnia; nightmares; Tension; Emotional lability; headache; tremor; apathy; Anxiety; Excessive dreaming; Withdrawal & depression; Bursts of slow waves of elevated-voltage in EEG especially on overventilation; Drowsiness; Difficulty concentrating; Confusion; Slowness of recall; Slurred speech; Ataxia; Generalized weakness; Convulsions; Coma, with absence of reflexes; Cheyne-Stokes respiration; Depression of respiratory and circulatory centres with dyspnea, cyanosis, and fall in blood pressure.

Table VIII (Leopold)



sure changes occurring in the erythrocytes. Blood cholinesterase activity has been shown to be increased (Gallois & Herschberg 1947; Vanysek 1948) and serum cholinesterase activity decreased (Arato & Arato 1952) in glaucoma patients. Recent well controlled studies employing the newer standard technique are equally conflicting (Dienstbier 1958; Leopold et al. 1959). Dienstbier could not demonstrate any appreciable difference between the red blood cells cholinesterase activity of normal and glaucoma subjects. Leopold et al. found the red blood cells cholinesterases levels to be slightly but significantly lower in glaucoma subjects as compared to the normals. Further studies in progress at present employing a different technique seem to fall more in line with Dienstbier's findings. Perhaps the level varies with the stage of glaucoma. The reasons for such a discrepancy in the results of various workers have been discussed by Leopold et al.

It may be concluded that at present there is no convincing evidence of a systemic disturbance of acetylcholine cholinesterase mechanism in glaucoma (See graphs I and II).

Apart from an attempt to incriminate systemic ChE in glaucoma, studies have been carried out to find any disturbance of acetylcholine-cholinesterase mechanism of the ocular tissues and fluids of glaucomatous patients. As has already been pointed out, conclusions based on any determination of ChE activity of the aqueous humor are misleading. Manometric determinations of ChE in the glaucomatous eyes have been attempted (DeRoeth 1950). All the eyes utilized in his study were absolute glaucomatous eyes representing the terminal phase of the disease accompanied by secondary changes. Thus, in both glaucomatous eyes and degenerated eyes, ChE contents were found to be diminished. If any conclusions are to be drawn from such a study, numerous glau-

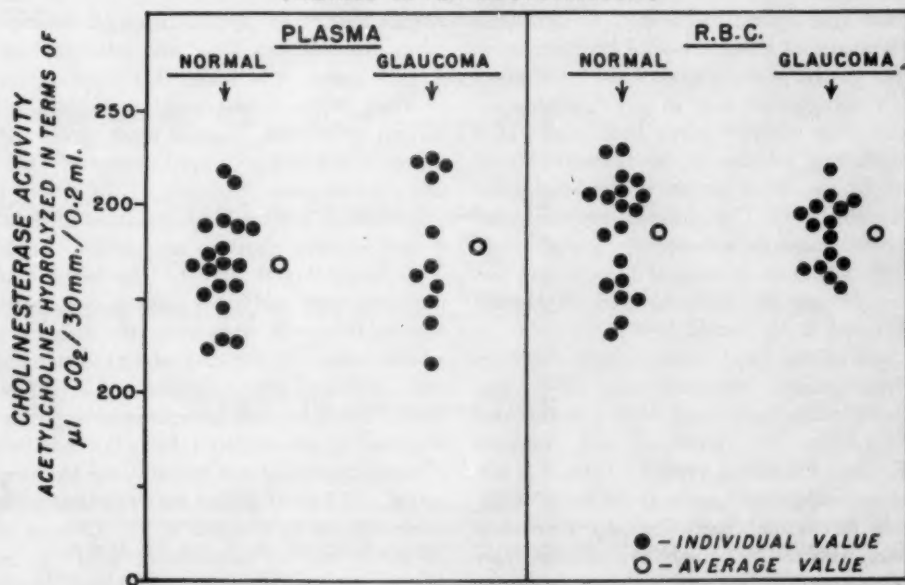
comatous eyes in various stages of the disease will have to be studied which is extremely difficult because of lack of material. Attempts at histochemical localization of ChE in such eyes may prove more rewarding. Thus there is no established evidence at present of any local disturbance of acetylcholine-cholinesterase mechanism in glaucomatous eyes.

Differences in Action of Anticholinesterases: Although many drugs are classified as anticholinesterases, they are not all similar in their activities. Some are short-acting and reversible; others have long action to the point of irreversibility; one may work well on myasthenia gravis; others not so well, or at all. These compounds differ in physical qualities such as solubility and, as we have seen, may inhibit different types of cholinesterase (See Table IX).

Interaction of Cholinesterase Inhibitors In Vivo: It is possible to demonstrate that one

anticholinesterase can protect the enzyme from another and also that one anticholinesterase agent may potentiate the activity of another. Prostigmine, for example, and DFP compete for the active surface of acetylcholinesterase. Prostigmine is a reversible inhibitor and DFP is considered to be an irreversible one. On incubation of a solution of AcChE with prostigmine prior to the addition of DFP in equal molar concentrations, it was found by Nachmansohn and Rothenberg that in dilution no inhibition had taken place. In the control experiment without prostigmine, the same molar concentration of DFP under the same experimental conditions had inactivated 50 per cent of the enzyme. The protection afforded by prostigmine against the action of DFP indicated that the two types of inhibitors were competing for the same site in the enzyme surface. Eserine also protects against DFP but not quite as efficiently as prostigmine.

**COMPARISON of BASE LINE PLASMA AND RBC CHOLINESTERASE LEVELS
IN NORMAL AND GLAUCOMA PATIENTS
(METHOD AFTER AUGUSTINSSON)**



Graph II (Leopold)

LOCAL OCULAR ANTICHOLINESTERASE AGENTS.

	ESERINE Physostigmine	NEOSTIGMINE Prostigmine	MINTACOL Paraoxon	BC 48 (Humorsol)	DFP Floropryl	PHOSPHOLINE (Echothiophate)
DURATION of MIOSIS	HOURS 12-36	HOURS 6-24	DAYS 1-3	DAYS 1-7	DAYS 1-14	DAYS 1-14
SIDE EFFECTS	-Browaches - Headaches - Ciliary spasm - Provocative rise in ocular tension - Cyst formation - Retinal detachment - Conjunctival irritation - Hypersensitivity - - Systemic effects.					
VEHICLE	WATER	WATER	WATER	WATER	ANHYDROUS	WATER
STABILITY	MONTHS	WEEKS	MONTHS	MONTHS	MONTHS	WEEKS
INHIBITS:						
Specific CHE	++	++	+	++	+	+
Non - Specific CHE	++		++		++	++
OUTFLOW MECHANISM: Increase Facility.	+	+	+	++	+	++

Table IX (Leopold)

An important practical consideration in ocular therapy is the synergism and antagonism exhibited by the various anticholinesterase agents used in the eye. Koster demonstrated that a small dose of physostigmine given to cats protects against the fatal action of a subsequent dose of DFP whereas a small dose of DFP given first results in a long-lasting increase in the susceptibility of cats to the lethal action of physostigmine (Koster 1946). The blocking mechanism of physostigmine to subsequent instillation of DFP on miosis in normal human eyes has been shown by Leopold and McDonald (Leopold & McDonald 1948).

Synergisms and antagonisms between physostigmine, demercarium, DFP and echothiophate have been studied in the eyes of rabbits by Krishna and Leopold (Krishna & Leopold 1960a) (Table X). Using subconjunctival route as the mode of administration and physiologically equivalent concentrations of these anticholinesterase agents, an attempt was made to determine the time interval required to cause the death

of rabbits following the administration of these agents. If physostigmine is injected first, followed very shortly by one of the anticholinesterase agents, namely neostigmine, demercarium, DFP and echothiophate in each rabbit, it is found that the duration of fatal period is shortened in rabbits receiving subsequent doses of neostigmine and demercarium and prolonged in case of DFP and echothiophate. However, if the order of injections is reversed, the duration of fatal period remains short in all rabbits (Table X). From this it may be concluded that physostigmine and demercarium are additive no matter in what order the drugs are administered. In the case of physostigmine and DFP, and physostigmine and echothiophate, the drugs are antagonistic if physostigmine is administered first, but additive if administered later. Clinically, the blocking action of physostigmine for DFP has been demonstrated by Comroe et al. (Comroe et al. 1946) and of neostigmine to DFP by Harvey and associates (Harvey et al. 1947) in patients with myasthenia gravis. The

blocking mechanism on intraocular pressure forms the subject matter of numerous studies on glaucoma. However, the evidence presented is equivocal as it is difficult to say whether the failure to bring down the intraocular pressure to normal is due to this blocking action or due to the refractoriness of the patient's glaucoma to the drug.

Attempts have been made to explain this blocking phenomenon. There is evidence to suggest that physostigmine, neostigmine and demercarium react reversibly with the same moiety of the ChE molecule as do DFP, TEPP, HETP, mintacol and echothiophate irreversibly. Koelle first reacted the brain ChE with various reversible inhibitors, then

added DFP and, after an interval, dialyzed the mixture. In the presence of physostigmine or neostigmine, the ChE was protected from inactivation by DFP (Koelle 1946). Perhaps in vivo this phenomenon may be explained in the following manner. When physostigmine is given first, it competes with DFP and ties up the AcChE in a reversible reaction and, during the time required to break this physostigmine-AcChE complex, most of the DFP is hydrolyzed and exerts no potentiating effect and after some time, when the reversible reaction between physostigmine and AcChE is over, there is no more DFP to tie up the enzyme. On the other hand, when DFP is given first,

COMBINATIONS OF ANTICHOLINESTERASE AGENTS.

Concentration of CHE Inhibitor		NO. OF RABBITS ↓	Average Time of Death AFTER 2nd. Injection (MINS.)
* 1st. Injection	♂ 2nd. Injection		
BC-48_____0.5%	Physostigmine_1.0%	6	3.5
Physostigmine_1.0%	BC-48_____0.5%	6	4.5
217 MI_____0.5%	Physostigmine_1.0%	6	4.5
Physostigmine_1.0%	217 MI_____0.5%	6	34.
DFP_____0.1%	Physostigmine_1.0%	6	4.5
Physostigmine_1.0%	DFP_____0.1%	6	105.

*=0.05 CC./LB. BODY WEIGHT SUBCONJUNCTIVAL INJECTION.

♂=2 ND. INJECT. GIVEN SUBCONJUNCT. 5 MIN. AFTER 1ST INJ.

Table X (Leopold)

it ties up first BuChE but also forms an irreversible combination with some of the AcChE. If physostigmine is given now, it still forms a reversible combination with the remaining AcChE. The blocking action in the case of echothiophate may be explained on the same basis. It may be important to realize that compounds like physostigmine, neostigmine and BC-48 react with the specific cholinesterase mainly while DFP, echothiophate, TEPP and other organophosphorus anticholinesterase compounds react with nonspecific cholinesterase predominantly and specific ChE to a lesser degree. The importance of this has not been evaluated fully in ophthalmology.

Thus from a practical point, if an organophosphorus cholinesterase inhibitor like

DFP, TEPP, HETP, mintacol or echothiophate is to be given to a subject who has already received physostigmine, neostigmine or demercurium, its administration should be delayed until the reversible inhibitor has been lost or excreted. If an additive effect is desired, it is recommended that the order be reversed, i.e. organophosphorus irreversible inhibitors given prior to use of the reversible cholinesterase inhibitors. Another way of obtaining the additive effect of course is the use of a combination of either reversible or irreversible anticholinesterase agents alone.

Mytelase and phospholine iodide have been shown to have a potentiating effect on their toxicity when administered peritoneally (Lehman 1959) (See table XI).

Combinations of these drugs when tried

ACUTE LD 50 RATIOS OF BINARY COMBINATIONS OF DRUGS IN MICE BY INTRAPERITONEAL INJECTION.

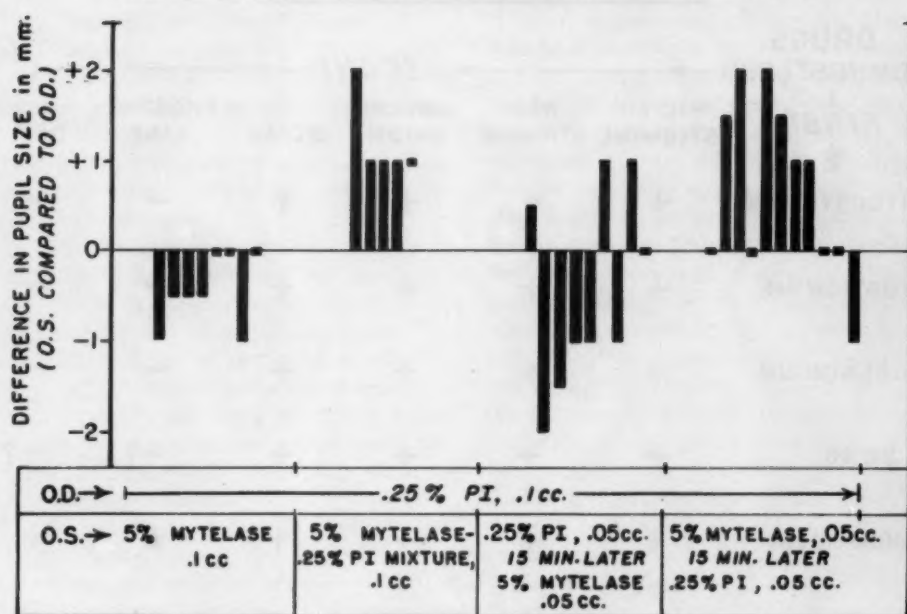
DRUG ↓	PROTECTION BY		POTENTIATION BY	
	PROTOPAM $\frac{1}{3}$ LD 50	TMB4 $\frac{1}{3}$ LD 50	PHOSPHOLINE $\frac{1}{2}$ LD 50	MYTELASE $\frac{1}{2}$ LD 50
	RATIO OF LD 50 WITH / WITHOUT OXIME #		RATIO OF LD 50 WITHOUT / WITH FIXED DOSE DRUG **	
Phospholine	24.	220.	—	53.
Neostigmine	2.2	4.2	4.6	1.5
Mestinon	1.4	3.0	0.7	3.3
Tensilon	0.9	0.9	0.9	12.
Humorsol	1.5	1.9	2.3	2.8
Mytelase	0.5	0.6	186.	—

POTENTIATION OF TOXICITY $< \frac{2}{3}$; ADDITION OF TOXICITY $> \frac{2}{3} < 1$;
NO EFFECT = 1; PROTECTION > 1 ;

** ANTAGONISM < 1 ; NO EFFECT = 1; ADDITION > 1 < 2; POTENTIATION > 2 .

Table XI (Leopold). After Lehman et al.

**PUPIL CHANGES INDUCED BY
COMBINATIONS OF PHOSPHOLINE AND MYTELASE.**



Graph III (Leopold)

locally for their effect on pupil size produce the results seen in graph III. Mytelase administered after phospholine has a slight but definite potentiating action. When phospholine was given after mytelase, no potentiation was encountered. Actually, a reduction of the miotic action of phospholine was observed. The influence of local combinations of several anticholinesterase agents on the pupil are summarized in Table XII.

Functional Adaptation to Cholinesterase Inhibitors: It is apparent that many eyes with chronic glaucoma appear to become resistant to continued use of anticholinesterase agents. This can be interpreted as representing an increase in severity of the glaucoma but it may be actually a functional adaptation of the tissues to cholinesterase inhibitors. Such adaptive changes have been observed in toxicity experiments, for cardiovascular effects and in neuromuscular trans-

mission by other observers (Paulet 1954) (Barnes 1954) (Barnes & Duff 1954) (Bombinski & DuBois 1958) (Krivoy & Wills 1958) (McNamara et al. 1954).

Use of the Newer Anticholinesterase Agents in the Management of Glaucoma: Following the original use of DFP for glaucoma by Leopold and Comroe in 1945, there has been considerable recorded experiences establishing this as an effective and useful drug in the medical treatment of specific types of glaucoma. More recently, several hundred eyes with glaucoma have been tested with phospholine iodide in concentrations ranging from 0.1 to 0.25 per cent. These are the results of many investigators (Leopold et al., Becker & Gage, Lawlor & Lee, Drance).

A similar number of eyes have been treated with BC-48 (See Chart VII) (Gittler & Pillat, Miller et al., Krishna & Leo-

COMBINATION OF ANTICHOLINESTERASE AGENTS LOCALLY ADMINISTERED

DRUGS ADMINISTERED	SECOND					
FIRST ↓	PHYSO- STIGMINE	NEO- STIGMINE	AMBEN- ONIUM	BC-48	PHOSPHO- LINE	DFP
PHYSOSTIGMINE	+	+	+	+	-	-
NEOSTIGMINE	+	+	+	+	-	-
AMBENONIUM	+	+	+	+	-	-
BC 48	+	+	+	+	-?	-?
PHOSPHOLINE	+	+	+	+	+	+
DFP	+	+	+	+	+	+

+ = INCREASED ANTICHOLINESTERASE ACTIVITY.
 - = DECREASED " "

Table XII (Leopold)

pold, Becker & Gage, Drance). All of these authors are in agreement that both phospholine iodide and demecarium bromide are effective agents in the control of glaucoma. They appear to control intraocular pressure in a large percentage of eyes uncontrolled on earlier conventional glaucoma treatment. These are long-acting anticholinesterase agents which offer the advantage of not requiring frequent instillations to maintain the lowered intraocular pressure and they are both stable in aqueous solution. Phospholine iodide may require refrigeration to maintain potency longer than a few months.

Approximately 50 per cent of the eyes with chronic glaucoma which had failed to

respond to other medication have shown a response to these newer anticholinesterase agents alone. As therapy is continued for longer than six months, some eyes are no longer controlled by these agents.

Both phospholine iodide and demecarium bromide have disadvantages which have been noted with other strong miotics and one must pay attention to the possibility of systemic toxicity.

Both agents are to be avoided in angle-closure glaucoma unless there has been a previous functioning iridectomy.

Both agents should be avoided where parasympathomimetic agents are contraindicated such as bronchial asthma, spastic gas-

trointestinal disturbances, hypertension, myocardial infarction, Parkinsonism and the like.

Phospholine iodide is employed in 0.1 or 0.25 per cent concentrations. BC-48 is usually employed as a .25 to .5 per cent concentration but has been employed in concentrations as strong as one per cent. The more concentrated the preparation, the more likelihood of adverse side effects including systemic symptoms.

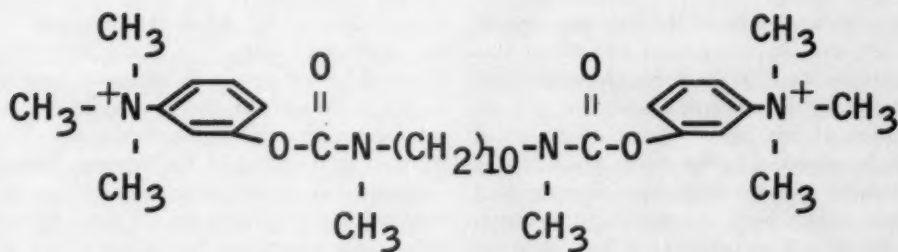
The frequency of instillation has to be determined on the individual case, preferably by observing the diurnal fluctuations as influenced by the drug administration. The concentration to be employed in each individual eye should be the lowest concentration which will bring about the desired result. There is a smaller percentage of failures, as one would anticipate, with the higher concentrations.

Ocular Effects of Locally Instilled Anticholinesterase agents: See Table XIII Effects on Extraocular Muscles—Electrical and Mechanical Activity: It is surprising that no studies have been reported on the effects of anticholinesterase agents on the extraocular muscles both in vitro and vivo

when applied locally, in spite of the fact that the extraocular muscles present such peculiar anatomical, physiological and pharmacological characteristics. Brown and Harvey who have studied the effects of intravenous and intraarterial injections of eserine on the extraocular muscles of decerebrated cats to some extent (Brown and Harvey 1941). After eserine has been injected, the extraocular muscles may show spontaneous contraction. The contraction produced by maximal single shocks applied to the motor nerve supplying the muscle is enhanced. Single nerve volleys produce a regular series of repetitive spikes which produces greater tension. Double nerve volleys have more complex effects, the eserine prolonging the refractory period of the muscle and interfering with the conduction in the nerve of the second succeeding volley. Eserine lowers the threshold of the curarized muscle to direct electrical stimulation. After eserine, both acetylcholine and repetitive nerve stimuli evoke a contracture which blocks the propagation of excitation along the muscle fiber.

Effects on Ocular Blood Vessels—Permeability of Blood-Aqueous Barrier: All anticholinesterase agents produce engorgement of

BC - 48 (Humorsol)



1, 10 Bis - (3 - trimethylammonium - phenoxy - N methyl carbamyl - N) - decane dibromide

Chart VII (Leopold)

OCULAR EFFECTS OF LOCALLY INSTILLED ANTICHOLINESTERASE AGENTS

Blood vessels —

Dilatation.

Permeability — increased.

Iris

Miosis.

Edema.

Ciliary body

Edema.

Accommodation — enhanced.

Outflow Mechanism

— Facilitated.

Intraocular Pressure

Early increase.

Late decrease.

Table XIII (Leopold)

the vascular supply of the anterior segment of the eye in both animal and man. This manifests itself in the form of conjunctival hyperemia, circumcorneal injection and dilatation of iris blood vessels, all of which can be observed in the living eye with the slit lamp and biomicroscope. Engorgement of the ciliary body and swelling and edema of the ciliary processes have been observed in the living eye (Matsuda 1959) following administration of physostigmine locally, and histologically following administration of DFP (Scholz 1946; VonSallmann & Dillon 1947); echothiophate and demercarium (Krishna & Leopold 1960a). The dilatation of capillaries profoundly affects the blood

aqueous barrier and the osmotic pressure of the aqueous (Barany 1947). The dilatation of blood vessels results in increased permeability to protein and causes a slight rise in intraocular pressure in the normal eye. The increase in turbidity of the aqueous humor induced by increased protein content can be detected in the aqueous on slit lamp biomicroscopic examination. Instillation of physostigmine causes significant increase of protein content of the aqueous of rabbit eyes (Wessely 1913; Swan & Hart 1940). Increased permeability and definite increase of protein in the aqueous has been demonstrated in rabbit eyes following instillation of DFP (Leopold & Comroe 1946b; Scholz

1946; VonSallmann & Dillon 1947). Caselli showed that mintacol produced a marked increase in permeability of the blood aqueous barrier 30 minutes after instillation in the rabbit eyes (Caselli 1953).

Effects on Iris and Ciliary Body—Pupillary Reaction and Accommodation: The effects on the iris and ciliary body of the locally used anticholinesterase agents have been investigated in great detail both in animal and man.

Much information in the past has been gained on the iris and ciliary body by *in vitro* studies. In these experiments, fresh isolated pieces of these tissues are obtained and the effects of various anticholinesterase agents studied by means of various mechanical, electrical and photographic devices. Such experiments can be rightly criticized as unphysiological when one considers that these tissues have been deprived of their vascular and nervous supply.

Ever since the first studies of Fraser (Fraser 1863) on the effects of physostigmine (eserine) on the eye when applied locally and its use as a miotic to counteract the cycloplegic action of atropine by Argyll-Robertson (Argyll Robertson 1863), numerous reports, have appeared concerning its miotic action and induced accommodative spasm. Instillation of physostigmine into the conjunctival cul-de-sac of the human eye in the strengths of 0.1 to one per cent causes pupillary constriction and spasm of accommodation; miosis begins in a few minutes and is maximal in about thirty minutes; intense miosis persists for nearly twelve hours and some degree of miosis may last as long as several days. The spasm of accommodation starting nearly the same time as miosis is of a shorter duration. The intense accommodative spasm tends to disappear in about two hours, but tends to remain irritable so that the slightest effort on accommodation for looking at a near object results in a ciliary spasm. The minimum concentration necessary to produce minimal pupillary constriction in humans is of the order of one

drop of 0.01 per cent (Marui 1922) and, in rabbits, one drop of 0.01 per cent (Molitor 1936). In rabbits physostigmine after instillation into the conjunctival sac can be demonstrated in the iris and ciliary body for seven to eight hours and the duration of miosis corresponds to the duration of the presence of eserine in the iris (Schumacher 1956).

While the anticholinesterase agents have been shown to exert powerful miotic effect on the normal eyes, they fail to induce miosis in denervated pupils. Thus Anderson showed that physostigmine failed to constrict the pupils in cats after the ciliary ganglia were removed (Anderson 1904, 1905a, 1905b). Leopold and Comroe have confirmed this observation and shown it to be true for neostigmine as well (Leopold & Comroe 1945). DFP also acts the same way (Leopold & Comroe 1946b). It is assumed that, after degeneration following postganglionic denervation, there will be no acetylcholine at the nerve terminals to excite effector organs. Thus the anticholinesterase agents will have no effect when instilled into such eyes.

Effects on Intraocular Pressure—Aqueous Outflow: Local application of anticholinesterase agents into the normal animals and human eyes generally causes decrease in intraocular pressure. This decrease is usually of the order of a few millimeters of mercury. In the vast majority of cases, there is a transient increase of intraocular pressure before the eyes show evidence of decrease in intraocular pressure. The degree of intraocular pressure lowering effect and its duration are directly proportional to the concentrations of the anticholinesterase agents apart from the individual potency possessed by each drug. The transient increase appears to be related to the ability of these agents to produce hyperemia and vasodilation especially of the iris and ciliary body. The dilatation of capillaries results in a temporary increase in permeability of blood aqueous barrier with the appearance of protein in the aqueous and

increase in intraocular pressure as has been demonstrated in both animal and human eyes in numerous studies (Wessely 1913; Szasz 1931; Swan & Hart 1940; Leopold & Comroe 1946b; Barany 1947; VonSallmann & Dillon 1947; Caselli 1953; Krishna & Leopold unpublished data; Krishna & Leopold 1960a). The elevation of pressure in eyes with narrow angles of the anterior chamber may be secondary to a pupillary block due to the intense miosis. The small pupillary border rests firmly against the lens and allows the posterior aqueous humor to push the iris plane forward crowding and occluding the angle. Although the decrease in intraocular pressure by the anticholinesterase agents has also been suggested on the basis of increased blood aqueous barrier resulting in diminution in the formation of aqueous humor (Barany 1947), this is not borne out by numerous other studies. It is now generally agreed that all anticholinesterase agents lower the intraocular pressure by lowering the resistance to aqueous outflow or by improving the facility of aqueous outflow. The exact mechanism is however not clear. Two different views have been expressed to explain this mechanism of action. According to one, improvement in the facility of aqueous outflow is due to the mechanical action on the trabecular meshwork exerted by the anticholinesterase agents due to pull of the scleral spur by the contracting iris and ciliary body. This results in opening of the channels in the trabecular meshwork through which aqueous gets ready exit. According to the other view, the collector channels and aqueous veins peripheral to Schlemm's canal are dilated and thus the resistance to outflow is lowered.

Scleral rigidity may be altered by these organophosphorous ChE inhibitors.

Ocular Use of Anticholinesterase Agents in Accommodative Esotropia: Esotropia or convergent strabismus may be accommodative, nonaccommodative or a combination of the two. The nonaccommodative type and the nonaccommodative element of the com-

bined type, as the name implies, are not influenced by accommodation and their treatment therefore is mainly surgical supplemented by orthoptics when indicated. The accommodative type and the accommodative element of the combined type are caused by accommodative effort and are amenable to measures other than surgery. Miotics are indicated in addition to other usual measures in the type of esotropia characterized by abnormal accommodation-convergence ratio, especially if the esotropia is markedly greater for near than for distance. The use of miotics alone or its combination with bifocal lenses is necessary. The principle on which the use of miotics is based is as follows: Accommodative effort is initiated by central nervous system and this is accompanied by convergence. If there is less central effort, there would be less convergence. By the use of miotics in the eyes, accommodation is achieved peripherally without any effort on the part of the central nervous system and consequently no convergence. Thus the aim is to increase peripheral accommodation and decrease central accommodation which is accompanied by greater amount of convergence.

DFP is the most commonly used anticholinesterase agent employed for this purpose, although others like physostigmine, mintacol, etc., have been used from time to time (Abraham 1949, 1952; Costenbader 1953, 1954; Schlossman 1954; Malbran & Norbis 1955; Knapp & Capobianco 1954; Stephen 1958; Sternberg & Raab 1959). The newer anticholinesterase agents, demercarium and echothiophate, are at present under trial in various clinics and seem to compare favorably with DFP. The use of DFP has been found most encouraging in suitably selected cases. DFP in strengths of 0.01, 0.025, 0.05 and 0.1 per cent in peanut or sesame oil or in the form of ointment has been employed. A drop is put in the eye each day for about two weeks. If beneficial effect is noted, the drug is continued at reduced frequency of administration at the widest interval of time com-

patible with therapeutic response from once every other day to once a week. In several instances, the treatment has been continued over a period of two years. Pupillary or iris cysts occur with greater frequency in children (Abraham 1954) but their incidence can be reduced by less frequent instillations. Nearly all anticholinesterase agents have been found more effective than pilocarpine which was previously employed because of the greater degree of accommodative spasm induced by them.

Several investigators have found that water-soluble and stable anticholinesterase agents, demercarium (BC-48), phospholine iodide (echothiophate), and mintacol (para-oxon), are comparable to floropryl (DFP) in the therapy of accommodative esotropia (Miller; Dunlap; Apt). Apt has noted some resistance of the child's ciliary and iris muscle to BC-48.

Ocular Use of Anticholinesterase Agents in Myasthenia Gravis: Myasthenia gravis, a disease characterized by weakness and paralysis of skeletal muscles, is of particular interest to the ophthalmologist as, in nearly half the patients, the initial symptoms are referable to the muscles of the eye and eyelids. These symptoms are ptosis and diplopia (Mattis 1941; Walsh 1945). Later, in practically all patients, symptoms are referable to the extraocular muscles. Again, there is a type of myasthenia gravis termed ocular myasthenia in which only muscles of the eyes and eyelids are involved (Walsh 1948; Lisman 1949).

The pathogenesis of myasthenia gravis still remains obscure, although it is referable to acetylcholine-cholinesterase mechanism disturbance resulting in deficiency of transmission at the myoneural junction. While the unusual susceptibility of the extraocular muscles in this disease is difficult to explain with certainty, it may be postulated that it is due to cholinergic disturbance as the normal extraocular muscles are unusually excitable by acetylcholine, physostigmine, etc. (Feng & Li 1940; Brown & Harvey 1941). Thus

these muscles are unusually dependent on acetylcholine and any disturbance in acetylcholine-cholinesterase mechanism is bound to manifest itself first in these muscles.

The use of physostigmine as a likely drug for treating myasthenia gravis was suggested by Jolly (Jolly 1895), but it was left to Walker to try not only physostigmine (Walker 1934) but also neostigmine (Walker 1935) in treatment of myasthenia gravis. Systemic administration of these and several other newer anticholinesterase agents since then has become the standard procedure for the management of myasthenia gravis patients. Neostigmine intramuscularly and tensilon intravenously are also used as diagnostic tests for myasthenia gravis where a positive response characterized by improvement in ptosis and ophthalmoplegia confirms the diagnosis of myasthenia gravis.

While a majority of patients are well controlled on systemic administration of anticholinesterase agents, there are those who still complain of symptoms referable to their eyes in spite of the fact that they are free from symptoms in the rest of the body. This may be ascribed to the fact that either there is not enough penetration of the anticholinesterase agents in these muscles or there is an abnormally high accumulation of ChE which the administered drugs are unable to destroy. Recently AcChE has been demonstrated by histochemical technique in the motor end plates of the extraocular muscles of both the normal and myasthenia gravis patients (Cohen & Zacks 1959) whose ocular symptoms remained uncontrolled on systemic administration of neostigmine. The presence of AcChE in these patients strongly suggests that there is not enough penetration of neostigmine in the extraocular muscles to inactivate the enzyme or the unusually high accumulation of the enzyme if such is the case. Apart from the failure of enough penetration of the anticholinesterase agents in the extraocular muscles, there are patients whose symptoms are confined only to the

eyes, the so-called ocular myasthenia. It is logical that this latter group be treated by means other than systemic administration of anticholinesterase agents with their accompanying undesirable systemic side effects.

Recently local use of anticholinesterase agents in the form of instillation of drops or iontophoresis in the conjunctival sacs has been advocated in patients with ocular myasthenia and in conjunction with systemically administered anticholinesterase agents in patients whose ocular symptoms are not relieved by the systemically administered drugs alone (Damiani 1957; Leopold et al. 1960). Cases have been reported in which marked subjective and objective improvement has been noted following instillation of neostigmine five per cent aqueous solution and demercarium 0.25, 0.5 and 1.0 per cent aqueous solutions into the conjunctival sac from twice a day to every second day. Electromyographic studies on the muscles of the eye and eyelids both before and after instillation of these agents confirm the efficacy of these agents and this mode of administration by improved and persistent firing of motor unit potentials on maximum and sustained effort following the instillation in contrast to the early disappearance of motor unit potentials on maximum and sustained effort before the instillation. The local use of these anticholinesterase agents in conjunction with the electromyographic studies of these muscles presents an attractive possibility for its being used as a diagnostic test for myasthenia gravis.

Reactivators of Cholinesterase: There are times when one is anxious to reverse the activities of the anticholinesterase agents, particularly when extreme toxicity has been produced. The ideal way would be to remove the toxic agent. The mainstay of therapy to date has been atropine. Numerous chemicals have been suggested and investigated as to the antidotal power against intoxication with anticholinesterase agents. They include such diverse compounds as cholinesterase itself, reversible anticholinesterase agents, chlorin-

ated hydrocarbons, lanthane salts and vitamin B-12. The most important of these are atropine and the reactivators of inhibited cholinesterases. The basis for antidotes, other than atropine and similar anticholinergic drugs, is the theoretical possibility that a drug which reactivates the enzyme—if it does so soon enough—will be useful in therapy since, once the enzyme has been reactivated, it will perform its normal function of destroying the accumulated acetylcholine and the symptoms of poisoning should disappear. Another approach would be to find a drug which, when administered prophylactically, will circulate in the blood or in the ocular tissues and fluids and destroy the anticholinesterase agent before it has a chance to reach the receptor site.

Jandorf in 1951 found that hydroxylamine reacted rapidly and smoothly with organophosphorus inhibitors in solution under physiological conditions and detoxified the compound (Summerson, W. H.). The same year Wilson reported both the spontaneous and hydroxylamine enhanced reactivation of TEPP-inhibited enzyme. Following this line of research, Wagner-Jauregg and his group in 1953 studied the compounds known as hydroxamic acids. These were relatively nontoxic and their reaction mechanism with anticholinesterase agents has been extensively investigated.

In 1953, Wilson and Meislich reported that DFP inactivated-cholinesterase could be regenerated successfully by treatment with dilute solutions of certain hydroxamic acids in vitro. This was the first example of how inactivation of cholinesterase by a firmly bound compound like DFP could be overcome in vitro. In 1955 Davies introduced pyridine-2-aldoxime methiodide (PAM) as a reactivator. Even more active compounds have been introduced subsequently.

At one time, the binding between phosphoryl compounds and the cholinesterase of the body was thought of as an irreversible process which irretrievably inactivated the enzyme. It was assumed that the enzyme had

to be restored by protein synthesis of the body. This opinion, however, had to be abandoned for several reasons. It was found that, if the enzyme inhibitor complex was incubated for sufficiently long time, a certain reactivation of enzyme activity occurred. Different inhibitors react differently in this respect in that the phosphoryl residue is more or less easily split off from the enzyme. Furthermore, it was difficult to see how the often ephemeral phenomena of intoxication could be based on inhibition with the time factor in the order of protein replacement. From a chemical point of view, the inhibition reaction must be considered an irreversible one because the original reactants are not all recovered, the inhibitors being transformed into innocuous products if reactivation occurs. Pyridine-2-aldoxime methiodide has been the most extensively studied of all the antidotes to date. The monoxoximes and

di-oximes of the bis-pyridinium ions are, however, considerably more effective in reactivating ethyl and isopropyl phosphorylated acetylcholinesterase than any other known compounds and there is the possibility that compounds in this group will make PAM obsolete eventually. The antidotal action of PAM has been described in a number of papers by Kewitz and his coworkers and also by others. It is important to realize that the results obtained with one particular organophosphate are not applicable to other organophosphorates even when it forms the same type of phosphorylated enzyme (Hobbig, F.) See Chart VIII.

PAM is a quaternary ammonium compound which penetrates poorly from the bloodstream into the brain or into the eye when systemically administered. Given systemically, PAM should be able to overcome the peripheral effects of anticholinesterase

REACTIVATORS

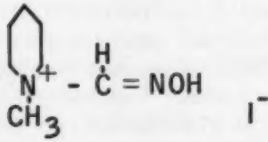
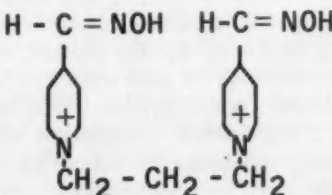
FORMULA	NAME	CODE NAME
H_2NOH	hydroxylamine	
$\text{CH}_3 - \overset{\text{O}}{\underset{\text{OH}}{\text{C}}} - \overset{\text{O}}{\underset{\text{OH}}{\text{C}}} - \text{CH}_2$	diacetylmonoxime	DAM
	pyridine - 2 - aldoxime methiodide	PAM 2 - PAM P - 2AM
	1,3 Bis (pyridinium-4- aldoxime) - propane dibromide	TMB - 4.

Chart VIII (Leopold)

compounds in addition to restoring neuromuscular transmission. It could not benefit effectively the poisonous effects in the face of continued central respiratory inhibition for it cannot reach this site. PAM is given either in repeated injections or in slow intravenous drip. 100 mg./kgm. of PAM may be given without untoward side effects. Systemic administration of these agents is indicated in systemic toxicity resulting from ocular use of anticholinesterase agents.

It is well known that the miosis resulting from systemic administration of anticholinesterase agents responds best to local administration of cholinergic blocking agents such as atropine, scopolamine, etc. and is not affected significantly by systemic administration of massive doses of atropine. Apart from this, there may be occasions when it may be desired to reverse the ocular effects of locally administered anticholinesterase agents. In nearly all instances, miosis can be counteracted by repeated and frequent instillations of one per cent atropine and four per cent homatropine solutions. With reversible inhibitors like physostigmine, prostigmine and demercarium, the result may be achieved in a shorter period than with irreversible inhibitors like DFP, TEPP, mintacol and echothiophate. Oximes and hydroxamic acids have been investigated from the point of view of counteracting the miosis induced by locally applied anticholinesterase agents when administered locally into the eyes (Mamo & Leopold 1958). Pyridine-2-aldoxime methiodide or methanesulphonate (PAM; 2-PAM; P-2 AM; P-25); diacetyl monoxime (DAM; DAMO); 1,3-Bis-(pyridinium-4-aldoxime)-propanedibromide (TMB-4); Monoisonitroso acetone (MINA); and 1,1-trimethylenebis (4-formylpyridinium oxime bromide) were the reactivators employed and physostigmine, DFP and echothiophate, the anticholinesterase agents employed. Administration of the oximes and hydroxamic acids into the cul-de-sac or anterior chamber of eyes alone does not produce any change in the pupillary

size of rabbits and normal humans, unlike atropine and homatropine which cause marked dilatation of the pupil. Administration of these agents locally after miosis has been induced by anticholinesterase agents results in reduction of the miosis. Local instillations of the oximes and application of ointments are not effective. Subconjunctival injections of five per cent 2-PAM are effective in counteracting the miosis induced by these anticholinesterase agents.

Although the initial studies with subconjunctival injection of 2-PAM were consistent in reversing the miotic effects of DFP, eserine and echothiophate, subsequent experiments have provided variable and inconsistent results (Mamo & Leopold 1958; Leopold & Krishna 1960) See graph IV.

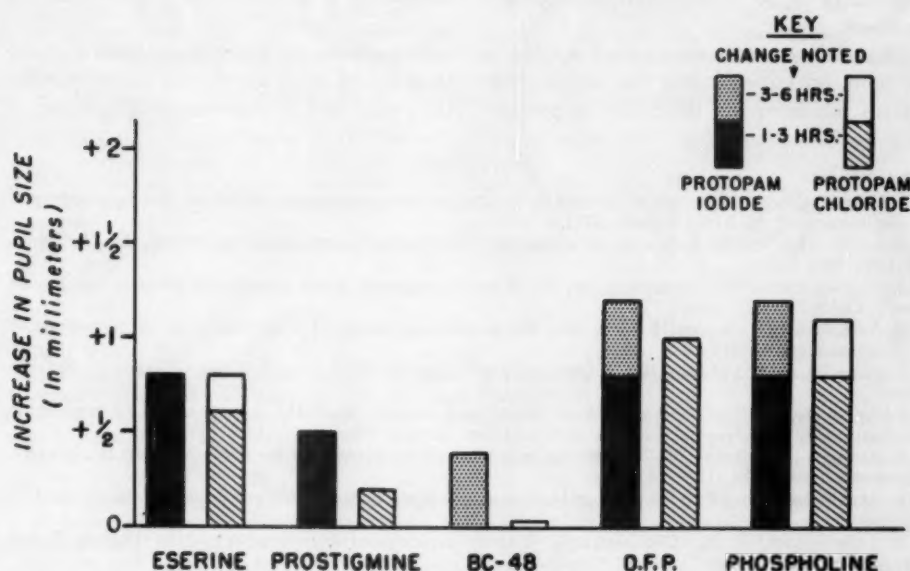
The miosis of eserine does not always respond nor does that of DFP or echothiophate. The reason for this is not apparent and requires further study.

BC-48 induced miosis does not respond to 2-PAM given subconjunctivally. Eserine is not an organophosphorus compound and is not expected to be reversed in its anticholinesterase activity by oximes such as 2-PAM or TBA/4. However, it was in the initial experiments and this has also been reported by Grob.

Reversal of the tonographic effects of topical echothiophate by 2-PAM have been studied (Becker & Pyle 1959). Subconjunctival administration of four per cent 2-PAM solutions in normal and untreated glaucomatous eyes produces no change in pupillary size, intraocular pressure and facility of aqueous outflow. In glaucomatous eyes previously treated with echothiophate, the miosis is counteracted and pupils are dilated, there is increase in intraocular pressure and decrease in facility of aqueous outflow, approximately reaching the same levels as prior to echothiophate administration. Thus oximes like 2-PAM when administered subconjunctivally counteract the actions of locally administered anticholinesterase agents such as miosis, decrease in intraocular pres-

EFFECT OF SUBCONJUNCTIVAL PROTOPAM AFTER ANTI-CHOLINESTERASE AGENTS

(RABBIT EYES)



Graph IV (Leopold)

sure and increase in aqueous outflow facility. 25 per cent solutions of 2-PAM chloride will occasionally penetrate sufficiently when instilled locally into the cul-de-sac to overcome pupillary effects of echothiophate. Ten per cent solutions of TMB/4 in a vehicle containing a detergent such as sodium lauryl sulfonate will also penetrate when locally applied. Such a detergent must be present in the vehicle to temporarily break the epithelial barrier to penetration. TMB/4 systemically administered as 25 mg./kg. close to the lethal dose will partially overcome pupillary effects of locally instilled echothiophate. The structure of these oximes apparently interferes with their arrival at the site of anticholinesterase-cholinesterase union when used topically or administered systemically. The local administration of oximes and hydroxamic acids to counteract local effects of anticholinesterase agents presents interesting

possibilities but at present their value is limited because their effectiveness demands the subconjunctival route and they are not uniformly and consistently effective.

SUMMARY OF ANTICHOLINESTERASE AGENTS IN OPHTHALMOLOGY

The organophosphorus agents with anticholinesterase activity have become a definite part of ophthalmic therapy. They may inhibit specific as well as nonspecific cholinesterase and differ significantly in their individual ocular actions.

The cholinesterase enzyme has been localized in ocular tissues of the experimental animal, but not by the most recent method. There is a need for such studies in normal and pathologic human eyes—particularly those with glaucoma.

The anticholinesterase agents that act on acetylcholinesterase reversibly may protect

the enzyme from the more prolonged action of the organophosphorus inhibitors.

The concept of the dual localization of the enzyme within and without the cell membrane may be of ocular pharmacologic importance.

The cholinesterase reactivators appear to be most effective against the organophosphorus inhibitors but their lack of penetra-

bility at present appears to reduce their therapeutic potential in ophthalmology.

The organophosphorus inhibitors are useful in ophthalmology in disease entities such as glaucoma, accommodative esotropia and myasthenia gravis.

Side effects are a prominent feature of the local use of these agents and ocular instillations may lead to systemic complications.

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GROWTH OF TOXOPLASMA STRAINS IN TISSUE CULTURE

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Toxoplasma has kept as a secret the methods by which it spreads to produce widespread disease in humans, as well as in domestic and wild animals. The genesis of the disease in the congenital form of toxoplasmosis is even more mysterious for it would be expected that a simple cause and effect relationship would exist between the disease in the mother and in the child. However, most studies have failed to reveal the method of spread of this disease to the fetus from an apparently healthy mother. At the present time it is not established that human toxoplasmic infections are acquired through insect vectors, airborne infections, by direct contact, or by food.

The high prevalence of positive skin and methylene blue dye tests in humans over age 20 in many countries, however, suggests a simple method of spread. The greater prevalence of positive tests in such countries as Guatemala, Tahiti, and other subtropical areas, and the lesser prevalence in arctic and subarctic areas such as Alaska, is not explained.

Considerable evidence is accumulating with respect to the organism, *Toxoplasma*. It is accepted that only one species of this organism exists, and that it belongs among the Protozoa, although its exact position among the Protozoa is uncertain. The variation in virulence of some strains of *Toxoplasma*, and the low virulence of many strains isolated from healthy animals has aroused the curiosity of many investigators. Erichsen and Harboe^{1,2} in Norway recov-

ered strains from chickens during an epidemic in a flock. They were later found not to produce appreciable disease in the progeny of the same flock. Jacobs and co-workers³ isolated a low-virulence strain from a pigeon and passed it repeatedly both in fertile eggs and in mice. The strain which was continuously passed in eggs retained its low virulence, whereas the one passed in mice became quite virulent. Lainson⁴ recovered six strains from rabbits which were of low-virulence for mice. Passage of two of these repeatedly in mice for over one year failed to increase their virulence appreciably. Chance passage both to multimammate rats and canaries caused a several fatal disease. Repassage of the same strain back to mice from the rats and canaries then caused marked and fatal infections.

A study of the growth of a number of strains of *Toxoplasma* of varying virulence in tissue culture cell lines seems to be a good approach to a solution of some of the mechanisms involved in toxoplasmic infections.

METHODS

Five strains of *Toxoplasma gondii* were selected (table I): RH, of high virulence for humans, mice, rabbits, and other animals; MF, a strain of very low virulence for laboratory animals which was recovered by us from a human congenital eye infection; CE113, a low-virulence egg-passaged strain originally recovered from a pigeon by Jacobs and Melton;⁵ JQ, a strain of very low virulence which was recovered by us from a human lymph node; and HI, a highly virulent strain isolated by us from a human lymph node.

The strains were grown on the following cell lines: HeLa; retinoblastoma (RB); human nasal epithelium (NaEp); adult rabbit kidney (SRK); embryonic rabbit kidney (ERK); and rat embryonic fibroblasts

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TABLE I
STRAINS OF TOXOPLASMA

NAME	ORIGIN	VIRULENCE FOR MICE
RH	CHILD ENCEPHALITIS	VERY HIGH
MF	20-YEAR-OLD HUMAN EYE	VERY LOW
113CE	PIGEON	MODERATE
HI	HUMAN LYMPH NODE	HIGH
JQ	HUMAN LYMPH NODE	VERY LOW

(LE). The characteristics of these cell lines are shown in Table II.

All cultures were prepared as follows: A cover glass of 10 x 30 mm. size was placed

on the flat side of a Leighton tube. The cells in stock culture were trypsinized and suspended in nutrient medium to obtain a suspension of approximately 50,000 cells/ml.

TABLE II
CELL LINES USED FOR THE EXPERIMENT

NAME	ORIGIN	CELL TYPE	CELL SIZE	GROWTH RATE MULT. /WEEK
HELA	HUMAN CERVICAL CARCINOMA	EPITHELIAL- LIKE	20 - 70u. IN DIAMETER	10
RB	HUMAN RETINO- BLASTOMA	FIBROBLAST- LIKE	40 x 80u. to 150 x 400u.	2 (IN 2 WEEKS)
NAEP	NORMAL HUMAN NASAL EPITHELIUM	EPITHELIAL- LIKE	30 - 70u. IN DIAMETER	3
SRK	ADULT RABBIT KIDNEY	FIBROBLAST- LIKE	40 x 80u. to 150 x 400u.	5
ERK	EMBRYONIC RABBIT KIDNEY	EPITHELIAL- LIKE	20 - 40u. IN DIAMETER	10
LE	RAT EMBRYO	FIBROBLAST- LIKE	10 x 60u. to 30 x 120u.	20

One ml. of this suspension was placed in the Leighton tube. The cells were incubated at 37°C for at least 20 hours before inoculation with *Toxoplasma*. Nutrient media used for the cultures consisted of 10 per cent dye test negative human serum in medium 199 (chemically defined medium) for HeLa and RB cells; 10 per cent calf serum in medium 199 for LE and ERK; 10 per cent calf serum in Eagle's basal medium for NaEp cells; and Imagawa's medium for SRK cells.

Three to seven-day mouse peritoneal fluid containing the various *Toxoplasma* strains was used as the inoculum. The number of organisms was counted in a hemocytometer and diluted in medium 199 to obtain a suspension containing ten times as many organisms per ml. as needed in the culture. One-tenth ml. of the suspension was inoculated into the Leighton tube cultures. The nutrient medium was changed twice a week.

For straining, the cover glass was removed from the Leighton tube, gently rinsed in saline, and fixed in Zenker's or Bouin's fixative for about four hours, then stained.

RESULTS

In order to make a comparative study of the early and late growth of the various *Toxoplasma* strains in the tissue culture lines, coverslips were removed, fixed and stained with Giemsa at 24 and 72 hours, and 7 and 28 days.

Preliminary studies showed that in all the cell lines inoculated with the RH and HI strains, the number of infected cells and the degree of initial cell destruction was proportional to the amount of inoculum. Generally with the MF, 113CE and JQ strains, 20,000/ml. or more organisms were needed in order to obtain about the same number of infected cells as was obtained with 5,000 organisms/ml. of the RH strain. Comparative susceptibility of the cell lines to a high virulence (RH) and very low virulence strain (MF) is shown in Tables III A, B, and C. The virulent HI strain behaved much as the RH strain and the 113CE and JQ strains behaved as the MF strain.

RH Strain. Cultures of the RH strain in all cell lines showed single organisms and

TABLE III A
EFFECTS OF VARIOUS STRAINS OF TOXOPLASMA
ON TISSUE CULTURE CELL LINES

TOXO STRAINS		HELA CELL LINE				RB CELL LINE			
		DAYS				DAYS			
		1	3	7	28	1	3	7	28
RH 5,000/ml.	P	2+	2+	2+	1+	3+	3+	2+	+
	R	-	2+	2+	1+	-	3+	3+	1+
	C	-	-	+	1+	-	-	1+	3+
	CD	+	+	1+	2+	-	-	1+	2+
MF 20,000/ml.	P	3+	1+	-			3+	2+	
	R	-	+	1+			2+	2+	
	C	-	1+	3+			-	3+	
	CD	-	1+	1+			-	-	

TABLE III B

TOXO STRAINS		NAEP CELL LINE				SRK CELL LINE			
		DAYS				DAYS			
		1	3	7	28	1	3	7	28
RH 5,000/ml.	P	3+	3+	2+	3+	3+	3+	3+	1+
	R	-	2+	3+	-	-	3+	3+	2+
	C	-	-	1+	1+	-	-	3+	3+
	CD	-	+	2+	3+	-	-	1+	3+
MF 20,000/ml.	P		1+	2+	1+		-	+	2+
	R		+	-	-		+	-	-
	C		2+	2+	3+		2+	2+	3+
	CD		1+	1+	2+		1+	1+	3+

pairs of organisms in the cytoplasm of many cells at 24 hours after inoculation (figs. 1, 2). At 72 to 96 hours rosettes were evident in many cells (figs. 3, 4, 5). After 7 days the cells often contained cytoplasmic cysts. Cyst-formation was clearly seen and best developed at 2 to 3 weeks in the RB and SRK cells (fig. 6). We now have observed them to persist in some cells for as long as

9 to 12 weeks (fig. 7). Pairs of organisms and rosettes were seen in the SRK cell cultures throughout the culture period, whereas in the RB cells pairs and rosettes were not observed after 4 weeks.

A majority of the HeLa, NaEp, ERK, and LE cells were destroyed by the organisms before cyst-formation occurred. In these cell lines at 3 weeks or later we ob-

TABLE III C

TOXO STRAINS		ERK CELL LINE				LE CELL LINE			
		DAYS				DAYS			
		1	3	7	28	1	3	7	28
RH 5,000/ml.	P	2+	2+	2+	2+	3+	3+	1+	1+
	R	-	2+	2+	-	-	3+	1+	1+
	C	-	-	-	-	-	-	+	+
	CD	-	1+	2+	3+	-	1+	2+	3+
MF 20,000/ml.	P		+	2+	3+		1+	1+	
	R		1+	1+	-		-	-	
	C		2+	1+	-		-	-	
	CD		1+	1+	3+		2+	4+	

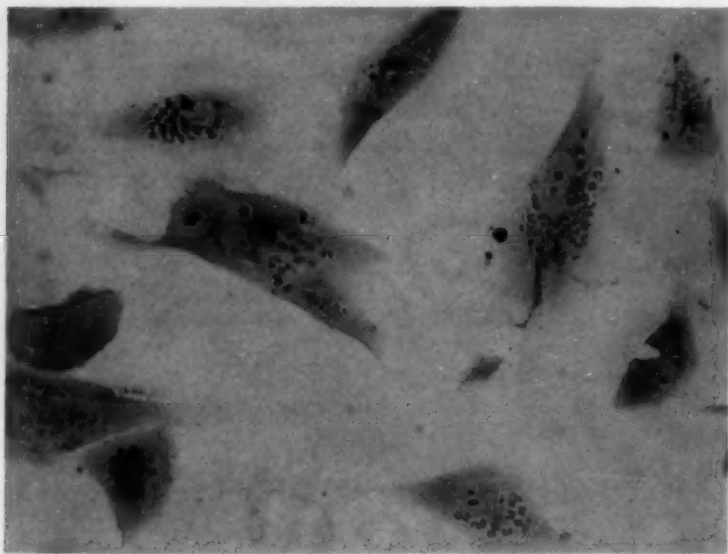


Fig. 1 (Hogan, Yoneda and Zweigart). RH strain in RB cell culture, 24 hours. Single organisms and pairs in the cytoplasm. ($\times 192$)

served many areas of dead cells which were surrounded by clusters of healthy cells containing pairs and rosettes (fig. 8). Rosette formation occurred in all cell lines inoculated with the HI strain. This strain regularly produced cysts in the cytoplasm of most cells after the seventh day. The number of cysts

seen at this time was much greater than in the cells inoculated with the RH strain.

MF, 113CE, and JQ Strains. Single organisms and pairs of organisms were seen in many cells of all cell lines in the first 24 hours of culture with these strains. Rosette formation occurred in all the cell lines inoc-

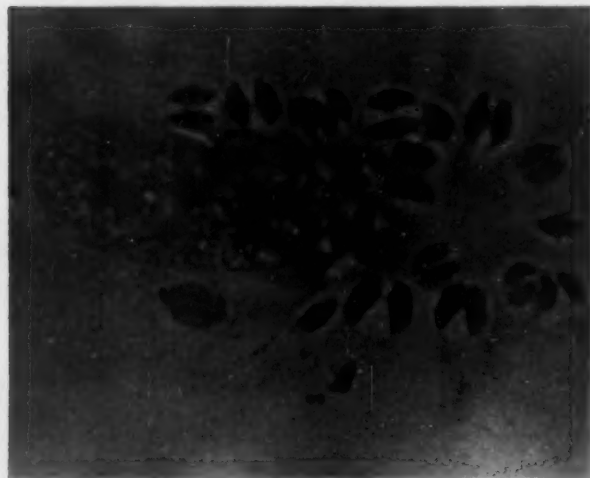


Fig. 2 (Hogan, Yoneda and Zweigart). Same as Figure 1. An RB cell containing pairs of *Toxoplasma* in vacuoles. These may not be true vacuoles; the contents around the organisms possibly have been removed during fixation and staining. Osmium-fixed electron microscopic sections show a smaller vacuole, and a fine stippled material in the space. ($\times 1400$)

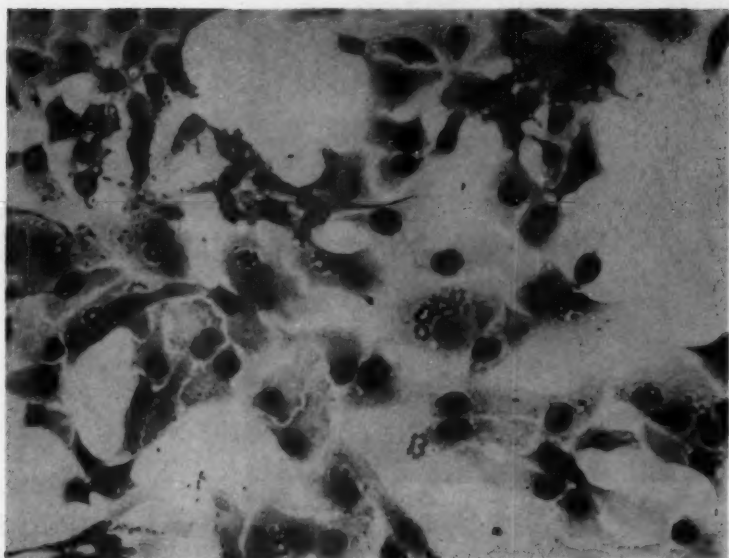


Fig. 3 (Hogan, Yoneda and Zweigart). RH strain in RB cells, 96 hours. Single organisms, pairs and rosettes. ($\times 576$)

ulated with the MF strain (fig. 9). However, they were fewer in number than in the cultures inoculated with the RH strain. No rosette formation was seen in any of the cell lines inoculated with 113CE strain.

Cyst-formation was observed as early as 72 hours after inoculation of the MF and 113CE strains into most cell lines (figs. 10, 11). These strains in the SRK cell line most often produced and maintained cysts longer than in HeLa, NaEp, or ERK cell lines. In all but the SRK cell lines, the cysts gradually disappeared by 2 to 4 weeks. At this time many zones containing dead cells were seen to be surrounded by healthy areas containing newly infected cells. It was noticeable that the principal areas of infection at this late period occurred adjacent to the originally involved cells. The organisms seemed to have spread to cells in the immediate neighborhood. Cells which were remote from the original zones of infection remained uninfected.

DISCUSSION

Toxoplasma has been cultivated in many

types of tissue culture with great success. Cook and Jacobs⁸ discuss publications on this subject up to 1958. Heavy inoculations (150,000 or more) of virulent organisms such as the RH strain invariably produce



Fig. 4 (Hogan, Yoneda and Zweigart). Same as Figure 3. Multiple rosettes in a single cell. ($\times 480$)

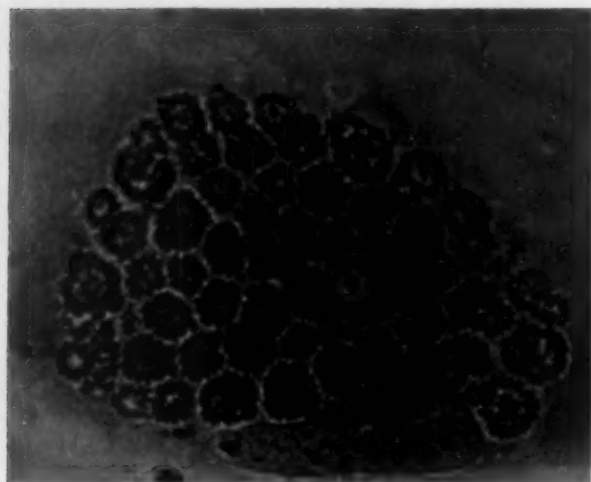


Fig. 5 (Hogan, Yoneda and Zweigart). RH strain in SRK cell culture, 4 days. Multiple rosettes in a single cell. ($\times 576$)

cellular degeneration and complete lysis of most explants and monolayer cultures within four to ten days, although Cook and Jacobs⁵ mention delay in cellular degeneration of cultures of conjunctival epithelium (Chang) and human intestine (Henle) beyond 12 days when infected with 200,000 RH organisms. We were able in this study, to maintain cultures in various cell lines for a longer period, probably because of the

smaller inoculum of organisms. Since this study was completed cultures have been maintained for 90 days, especially in the RB and SRK cell lines. It is interesting that localized areas of invasion and cell destruction occur, surrounded by cells which often are healthy, and contain no organisms.

The virulent RH organisms invade cell lines in tissue culture rapidly. Within a few minutes after inoculation a large number of cells become infected⁶ and multiplication of organisms commences. A number of organisms can invade a single cell and form localized colonies. Reinfection of cells can occur at any time, and within 24 to 48 hours one often sees a single cell with several pairs of organisms at one side, and a well-developed colony at the other.

The formation of rosettes is curious, and is a prominent finding in the cultures of RH and HI strains in RB and SRK cell lines. It is known that the organisms seem to lie in a vacuole in the cytoplasm of the cell.^{6,7,8,9} Division by binary fission results in pairs of organisms which are oriented in a single plane. The two organisms in the pairs always seem to be related to each other in the same way, suggesting that motion does not occur after division, or that they are so confined in the vacuole that they cannot change

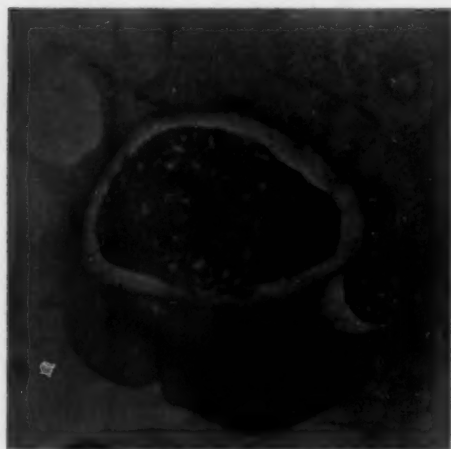
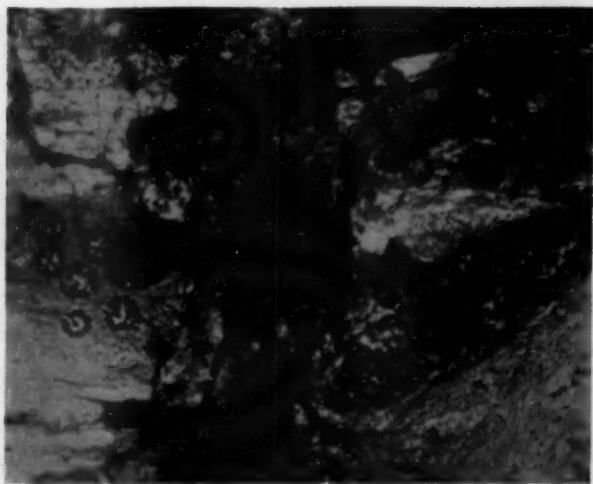


Fig. 6 (Hogan, Yoneda and Zweigart). RH strain in RB cells, 7 weeks. Two cysts in a single cell. The cyst wall can be seen best at the lower border of the large cyst. ($\times 576$)

Fig. 7 (Hogan, Yoneda and Zweigart). Late cyst formation of the RH strains in SRK cells (12 weeks). Cells showing cysts, rosettes and pairs of organisms. Note large cell containing a number of cysts. ($\times 365$)



relation to each other. Further division leads to formation of a layer of organisms all oriented in a single plane, and it must be that the rosette forms as a result of this division in a single plane.

Further proliferation of the organisms in a rosette often leads to formation of a ball-like structure. These occur later in growth of the cultures. We¹⁰ have shown by electron

microscopy that these are true cysts with a well-defined wall. Sourander et al.⁶ described a definite, dark boundary around the cells in the living as well as fixed state. These cysts develop by the seventh to fourteenth day of culture in some cell lines, and within three to seven days in others. The RB and SRK cells, which maintained the cysts longer, are large and slow-growing cells, which might

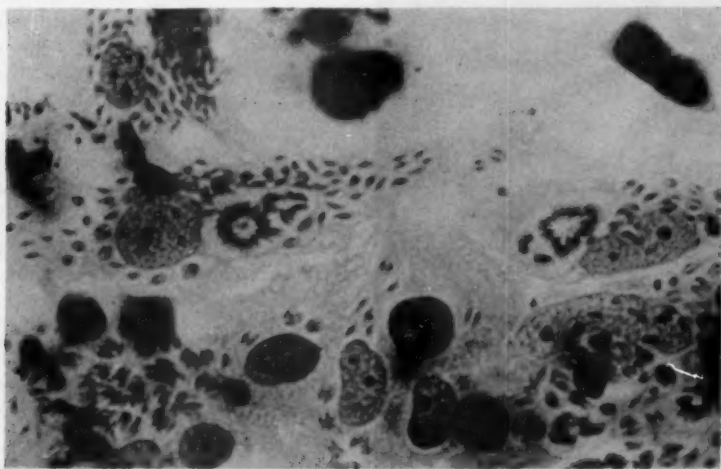


Fig. 8 (Hogan, Yoneda and Zweigart). RH strain in HeLa cells (3 weeks). Pairs and rosettes in a single cell at 3 weeks. Note the dead cell containing a rosette at the lower right. Many areas in this culture showed dead cells. ($\times 730$)

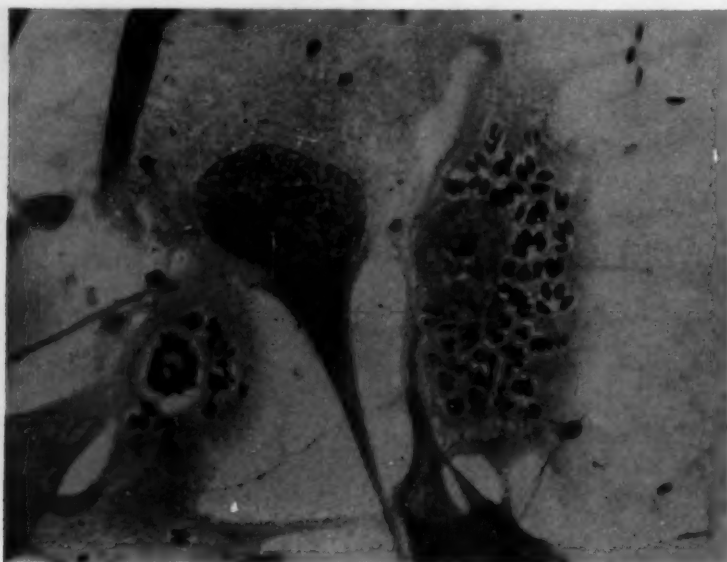


Fig. 9 (Hogan, Yoneda and Zweigart). MF strain in HeLa cells, 48 hours. Single organisms, pairs and rosettes. Note free organisms. ($\times 480$)



Fig. 10 (Hogan, Yoneda and Zweigart). MF strain in HeLa cells, 7 days. Cysts in cells containing small, rounded organisms. ($\times 575$)

account for their greater ability to produce the cysts. The HeLa, ERK and LE cells are smaller and proliferate more rapidly. Cysts were not maintained for very long in any one of these rapidly growing cell lines, except occasionally in the NaEp cells.

Kaufman et al.¹¹ studied the growth of three strains of *Toxoplasma* of varying virulence for mice in human amnion cell cultures. One was the virulent RH strain; another the moderately virulent 113Ce; and a less virulent strain, S₈. It was shown that virulence was correlated both with an increased proportion of cells invaded and a more rapid rate of multiplication of the organisms. Our studies would confirm this, for, as shown in the tables the virulence of the organisms played an important role in the degree of cell destruction and the time of cyst formation. The less virulent strains produced less destruction in most cell lines, and tended to produce cysts earlier. The rapidity of proliferation and the size of the tissue

culture cells seems to have some influence on the behaviour of the organisms in tissue culture, but the reason for this influence is not apparent.

It should be remembered, however, that most of the less virulent strains for mice become more virulent if they are passed rapidly. Also, that they proliferate more slowly and are fewer in number in mouse peritoneal fluid at four days than the more virulent strains. An occasional occurrence, such as the effect of the MF strain in the LE cell line, cannot be explained on the basis of virulence for mice.

SUMMARY

1. Five strains of *Toxoplasma* (one highly virulent; one of slightly lower virulence; and three of low virulence) were grown in six cell lines (HeLa; retinoblastoma; human nasal epithelium; adult and embryonic rabbit kidney, rat and embryo), for one to 28 days. Cultures could be maintained for the full period of the experiment and produced infection in mice after culturing for 28 days.

2. The virulent RH strain was invasive for all cell lines, often producing extensive cell destruction. Invariably it produced many rosettes within two to seven days after inoculation. An occasional cytoplasmic cyst developed in about seven days, but many more were noted after two weeks.



Fig. 11 (Hogan, Yoneda and Zweigart). MF strain in RB cells, 7 days. Cysts in the cytoplasm of a single cell. Also several larger recently divided organisms. ($\times 730$)

3. The less virulent strains, even though inoculated in larger numbers, most often produced less cell destruction. However, cyst formation occurred earlier with these strains.

4. The size of the cells and the rapidity with which they multiply and the virulence of the organisms all have an effect on the formation of rosettes and cysts. The virulence of the organism itself seems most important.

5. Rosette formation is a curious phenomenon, and probably is based on the method of division exhibited by *Toxoplasma*, and the restraint imposed on it by the cytoplasm.

San Francisco (22).

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THE EFFECT OF ENDOTHELIAL CURETTMENT ON CORNEAL WOUND HEALING: EXPERIMENTAL STUDY

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This paper concerns itself with the effect of endothelial curettment on the course of corneal wound healing in the rabbit eye. We have attempted to determine these effects in each component of corneal wound healing, that is the effect on the stromal, fibrin, epithelial and fibroblastic phases.

MATERIALS AND METHODS

Pigmented mongrel rabbits were anesthetized with intravenous sodium nembutol, retrobulbar novocaine and topical pontocaine.

Experiment I: In 17 rabbits a 5.0 mm. central penetrating corneal incision (of Sae-misch type) was made on each eye. Immediately after the incision the endothelium of the right eye was gently curetted with a small chalazion curette through this central incision. Only the wound edges in the left eyes were traumatized to correspond to the trauma incurred during curettment of the other eye. These served as controls. No sutures nor medications were used after the incision.

Experiment II: Both eyes of seven rabbits were similarly incised except that only the inferior portion of the right cornea and the superior portion of the left cornea were curetted. Thus the uncuretted side of the wound in each eye served as its own con-

trol. These were followed up to a period of one month by slit lamp examination, external photographs and histologic sections. Counts of epithelial cells, eosinophiles and fibroblasts were made on four sections of each eye as will be described.

ONE DAY

Experiment I: The stroma in the control eyes (fig. 1) showed a mild edema limited to the area adjacent to the wound and its fibrin plug was solidly formed either between the wound edges or in the posterior aspect of the wound edges. In all but one of 12 control eyes the anterior chamber was well formed. This exception had a shallow chamber in spite of a solid plug.

In contrast, those curetted corneas showed a persistence of the opaque stromal thickening about three times normal. The edema was mostly in the anterior one-fifth of the original stroma so that this part looked almost cystic in optical sections. The fibrin plug was grossly deficient in six of 12 eyes, loosely attached anteriorly in five others and almost complete in the remaining eye. In contrast to the controls the anterior chamber was flat in all of 12 eyes.

Experiment II: On the curetted side the cornea was opaque and thickened by edema while the control side was clear except for some edema about the wound (fig. 2). The fibrin plug seemed adequate in eight of 12 wounds, deficient in two and undescribed in

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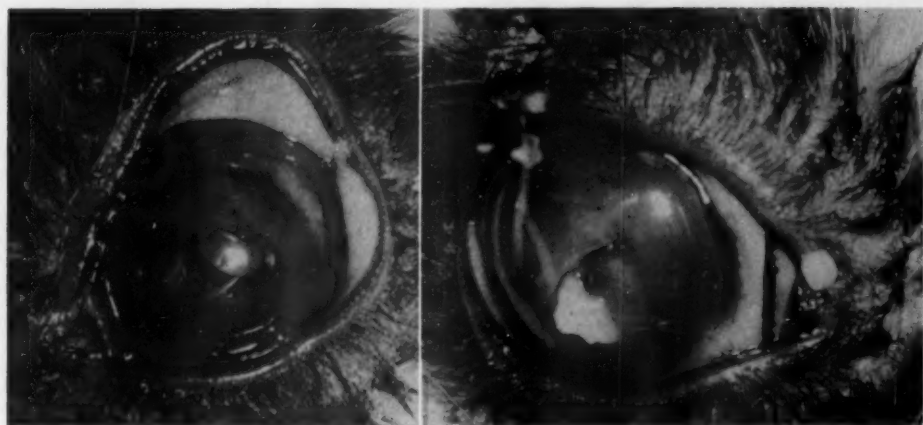


Fig. 1 (McDonald). Experiment I, one day postoperative. (Left) Control. (Right) Curettered.

two. In all 12 eyes the anterior chamber was flat on the curettered side while it was deep in nine, shallow in one and flat in two on the control side.

ONE WEEK

Experiment I: The control eyes were well healed, the cornea remained clear with little stromal edema in the region of the wound (fig. 3). In all cases the chamber was well formed. The plug was solid in all but one of eight eyes.

The curettered eyes differed in that the marked stromal opacity and edema persisted and the wound edges were more widely separated. In several of these eyes the plug seemed grossly deficient. The anterior chambers were well formed in all eight controls and flat in all curettered eyes except that two of these eight showed crevices or valleys of chamber formation.

One of these curettered eyes showed an enlargement of the cornea and was described as buphthalmic.

Experiment II: The opacity and edema of the cornea on the curettered side now showed a tendency to clear at its edges. The wounds were well sealed (fig. 4). The previously flat half of the iris began to form at its edges leaving evidence of pigment or fibro-

blastic opacification in the posterior cornea. In all cases broad anterior synechias remained varying from one-third of the cornea to an area of only one hour on the clock.

TWO TO FOUR WEEKS

The control eyes healed normally. The curettered ones showed the scarred and opaque cornea (fig. 5). Two of four wounds of the curettered eyes were well sealed; the other two were definitely fistulizing; two were buphthalmic, all anterior chambers were flat with some pockets of formation as in earlier studies.

In summary, observations with the slit lamp showed that curettement of the endothelium in Experiment I caused a marked

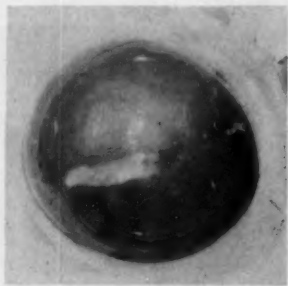


Fig. 2 (McDonald). Experiment II, one day postoperative. Upper one-half curettered.

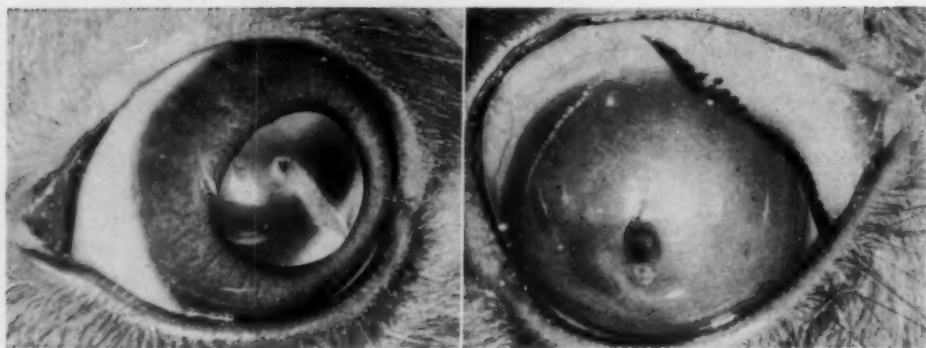


Fig. 3 (McDonald). Experiment I, one week postoperative. (Left) control; (Right) Curretted. Note wound gape and plug deficiency.

edematous thickening and later scarification of the stroma, an interference with the integrity of the fibrin plug, a persistent flat chamber, almost total anterior synechias and secondary glaucoma in some eyes. Experiment II showed stromal edema on the curretted side, little or no interference with the integrity of the fibrin or subsequent fibroblastic plug, a flat anterior chamber on the curretted side only and a pigmentation and scarification at the endothelial level and persistent anterior synechias.

HISTOLOGY

ONE DAY

The stroma over the curretted area showed a massive edema resulting in a thickening anteroposteriorly of two to three times normal (fig. 6A). This edematous stroma did not possess any tendency for abutment against the opposite side of the wound.

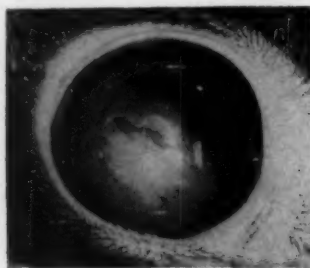


Fig. 4 (McDonald). Experiment II, one week postoperative. Lower one-half curretted.

Separation of the wound edges in the edematous cornea was greater than in the controls. Histologically, we could not confirm the slit lamp observation that the anterior portion of the cornea was more edematous than posteriorly. This may be due to fixation techniques.

The fibrin phase in the control was expressed histologically by adherence of the fibrin to the edges of the fractured lamellae, thus sealing the wound with no tendency of the fibrin to adhere wherever epithelium or endothelium was present. In contrast, curretted corneas showed a fibrinous adherence along the entire curretted portion as well (fig. 6A), causing an adherence of the iris to the posterior cornea. This resulted in a histologically flat chamber in the two curretted eyes from Experiment I, but a deep chamber in the controls. A flat anterior chamber resulted only on the curretted

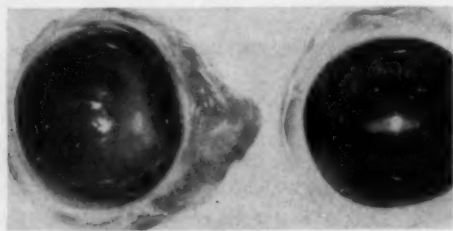


Fig. 5 (McDonald). Experiment I, two weeks postoperative. (Right) Control. (Left) Curretted.

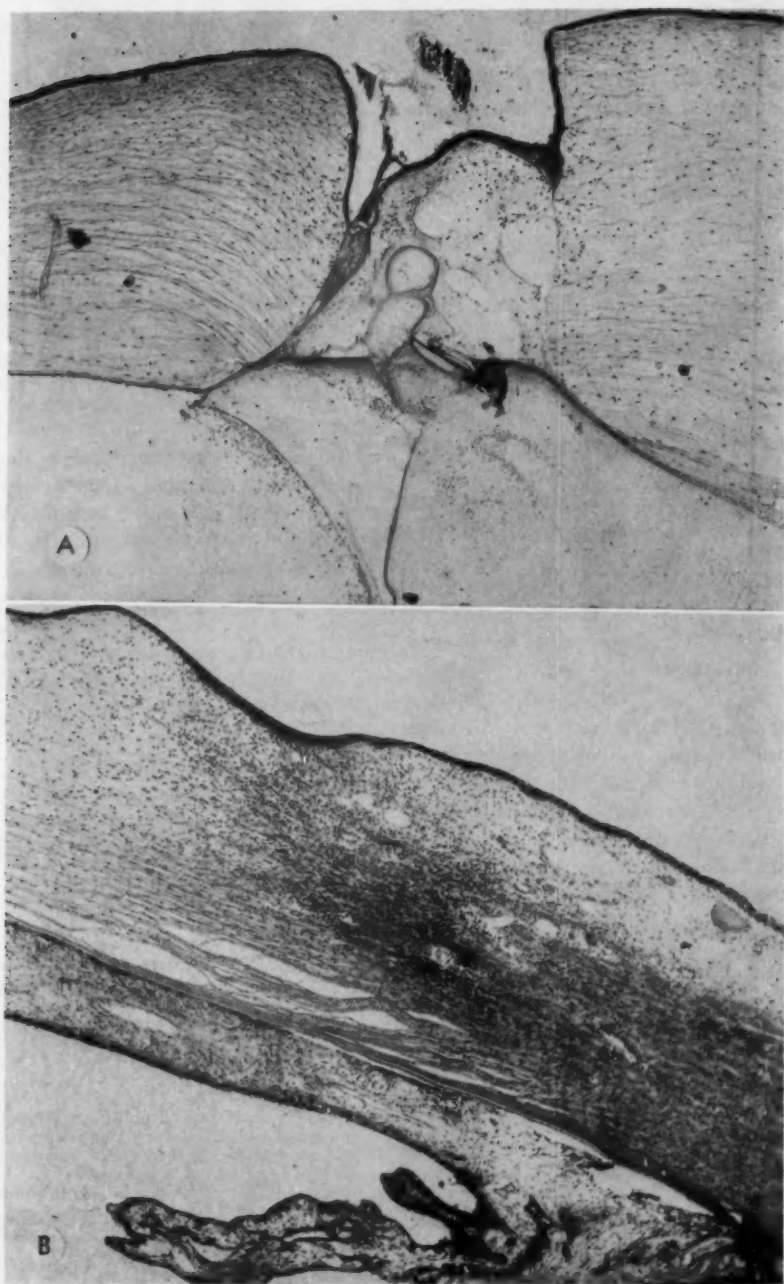


Fig. 6 (McDonald). Experiment II, one day postoperative. Top, right side curetted; Bottom, curetted side.

side in the two eyes of Experiment II (fig. 6B). It should be mentioned that this curettment of the endothelium resulted in the production of some areas of detachment of the Descemet's membrane in most eyes. Therefore, these experiments compare the effect on wound healing of normal endothelium in the controls on the one hand, with areas of endothelium or Descemet's absence and even of normal endothelium (areas where the curette missed the endothelium) on the other. These fibrin adhesions were most dense where Descemet's was missing compared to where only endothelium was missing.

All the epithelial cells which had migrated beyond the upper wound edges in Experiment I were counted on four sections each of two eyes and their controls one day postoperative. These counts showed no inhibition by curettment of migration of these cells (Table I). The epithelium in the curetted areas showed edema as manifested by an increase in size and less intense staining of the basal cell layer, a displacement of the nucleus away from the basement membrane and an enlargement and reduced staining of the nucleus in contrast to the compact, somewhat irregularly placed smaller dark-staining nucleus of the control. Our findings were similar to the mildest of the changes described by Cogan.¹ No gross bullae or subepithelial debris were seen.

The cellular elements in the stroma near the wound consisted of stromal cells (fibroblasts?) and eosinophilic, polymorphonuclear cells. At the stromal edge there was a deficiency of these fibroblast-like cells (fig. 7A). These findings are not incompatible with observations of Wolter^{2,3} and Weimar^{4-discussion}. This area of relative acellularity for this type of cell was about 0.1

TABLE I
EPITHELIAL CELL COUNTS*

	Rabbit	Curetted	Controls
1 Day	a	19	36
	b	28	20
3 Days	c	47	9
	d	43	15
4 Days	e	19	56
	f	41	24

* Epithelial cells beyond wound edges per millimeter length of wound, in thousands.

millimeter and was approximately equal in the control and curetted wound. Counts of these fibroblast-like cells in an area 0.3 millimeter from each wound edge showed a slightly greater number of these cells in the control eyes (Table II). These may indicate a greater necrosis of stromal cells near the wound edge of the curetted eyes, although only four sections of each of six eyes were counted at one day. The posterior five to six lamellae were relatively acellular over the curetted areas especially when Descemet's also was missing (figs. 7B, 7C).

On the other hand, counts of eosinophilic cells which infiltrate the same area of the wound, showed remarkable variation from only 30 cells per millimeter length of wound in one eye to 14,000 in another. The dis-

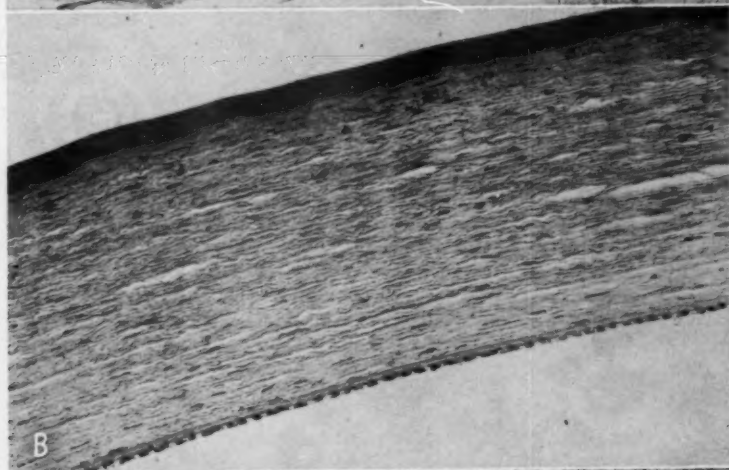
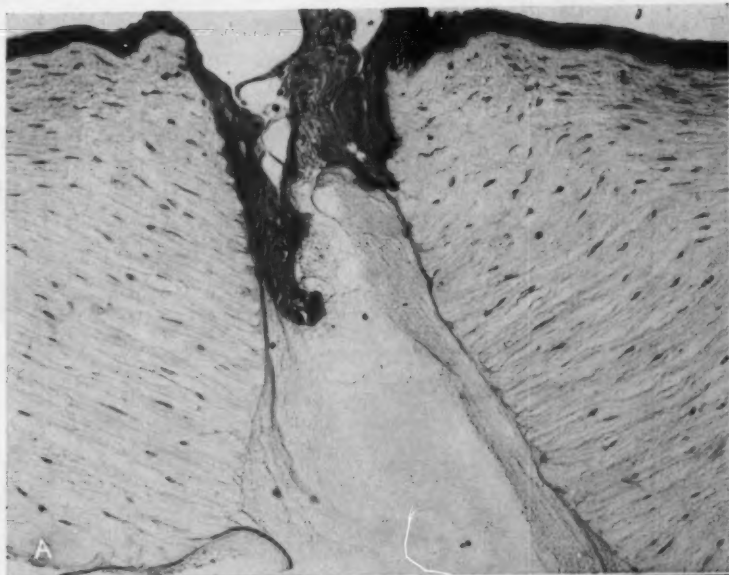
TABLE II
STROMAL CELL COUNTS

	Rabbit	Curetted		Controls	
		Eosin	Stromal Cells	Eosin	
1 Day	A	2	17	24	9.0
	B	7	16	19	0.03
	C	14	18*	22*	9.0

Only 0.3 mm. distance from wound edges included. Numbers show cells per millimeter length of wound in thousands. Only eosinophiles in stroma counted.

* From Experiment II curetted sides of both eyes added to obtain total for one curetted wound. Same with control sides.

Fig. 7 (McDonald). Experiment II, one day postoperative (high power). (A) Right side curetted. Wound showing paucity of stromal cells near wound edge. (B) Control side. (C) Curetted side. Note iris adherence and stromal cell paucity in posterior lamellae.



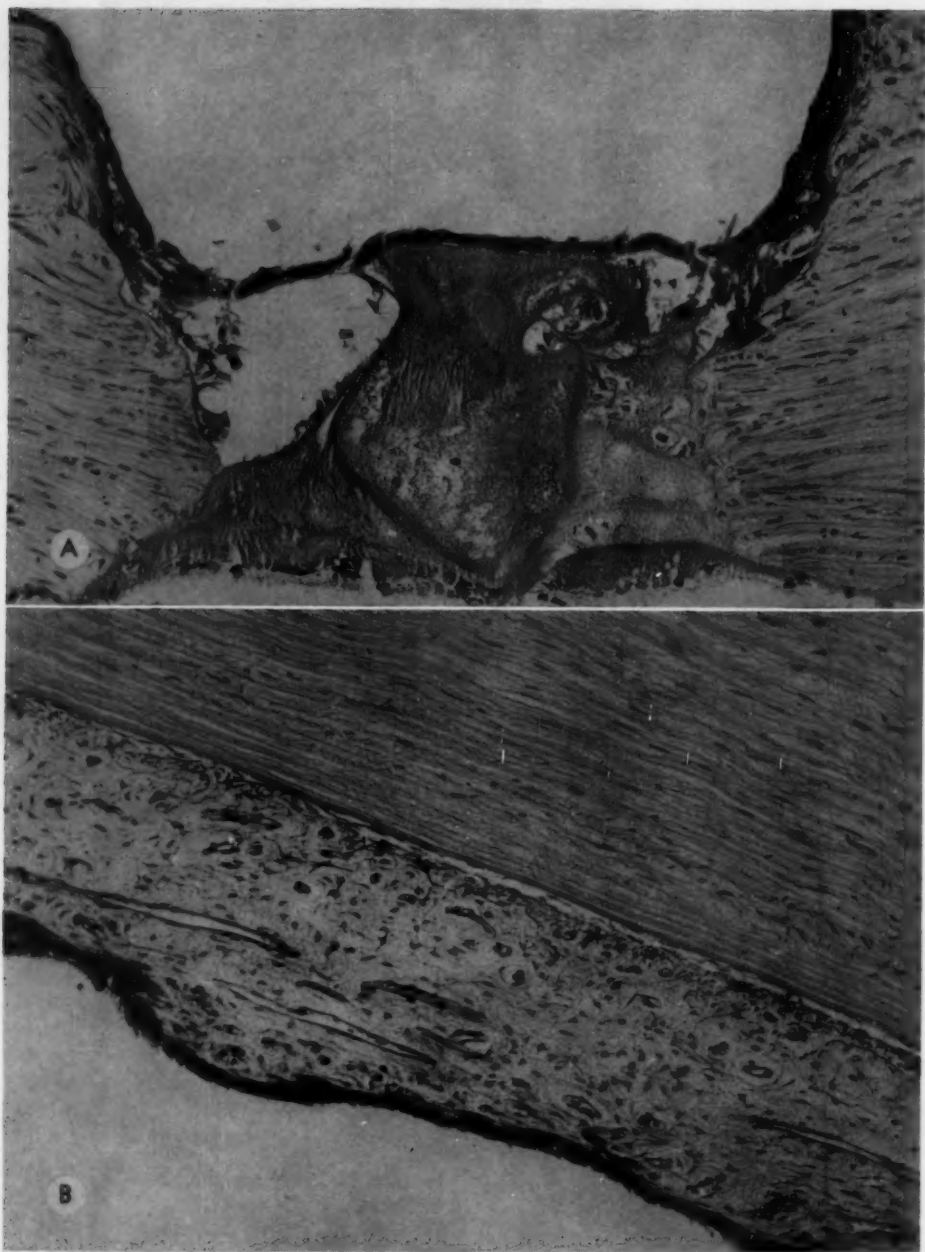


Fig. 8 (McDonald). Experiment II, three days postoperative. (A) Early fibroblast invasion of wound edge and fibrin plug are seen. (B) Cured side, iris adherence and paucity of stromal cells persists.

tribution of these eosinophils indicate that other factors are of greater significance in determining their number than the effect of stromal edema.⁸ There was no deficiency near the wound edge of the eosinophilic cells, in fact they seemed concentrated here as they migrated toward the fibrin plug.

THREE DAYS

At three days the histology differed in that the fibrin plug in the controls was more dense and its strands orientated more perpendicular to the wound edges. The areas of fibrin adherent to the posterior cornea correspond to the areas of curettement, these adherences being more dense in the area of Descemet's absence and not present in the area of healthy endothelium. As in the previous observations, these fibrin adherences have resulted in a completely flat chamber in the curetted eyes and a flat chamber only on the curetted side in Experiment II (figs. 8A, 8B).

Epithelial cells in the curetted eyes differed from the controls in the same way as described on the one day reading. Quantitation of epithelial migration at three and four days (Table I) reveals an inconsistent

increase in the number of epithelial cells in the curetted eye.

In both the controls and the curetted eyes cellular characteristics at the wound edge were now changed (fig. 8A). There was a substantial increase in the number of fibroblasts in the stroma (Table III), as well as those which had migrated beyond the stroma toward the plug. There were more fibroblasts in the curetted animals at three days but not at four days in comparison to the controls indicating at least that stromal edema does not inhibit fibroplasia at the wound edge and somewhat suggestive of an actual stimulation of fibroplasia, as suggested by Findley and Howes.⁶ There still remained a marked acellularity of the posterior stroma. As in the one day histology, a remarkable variation in the number of eosinophiles within the stroma. Eosinophiles within the fibrin plug were not counted but were now in greater number than at one day.

ONE WEEK

The decreased importance of fibrin as a sealer of the wound and adherer to tissues bared of their normal covering was evident

TABLE III

	Rabbit	Eosin	Curetted		Controls	
			Stromal Cells or Fibroblasts		Eosin	
3 days	D	0.5	(33) 40	32 (26)	30.0	
			(7)	(5)		
	E	0.8	(46) 55	35 (33)	0.3	
			(9)	(3)		
	F	0.8	(49) 60*	*46 (41)	0.7	
			(11)	(5)		
4 days	G	0.1	(43) 58	68 (43)	3.0	
			(14)	(24)		
	H	1.3	(37) 58	54 (27)	0.7	
			(21)	(27)		

Upper number in parenthesis indicates fibroblasts in stroma, lower those which have migrated beyond wound edge. Only 0.3 mm. distance from wound edges included. Numbers show cells per millimeter length of wound in thousands. Only eosinophiles in stroma counted.

* From Experiment II curetted sides of both eyes added to obtain total for one curetted wound. Same with control sides.

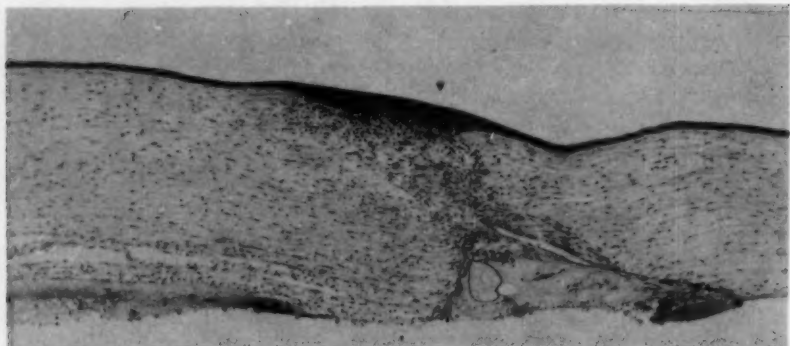


Fig. 9 (McDonald). Experiment II, one week postoperative. Left side curetted. Note increased fibroplasia of wound and curetted stroma and persistence of fibrin where curetted.

now. This material which still remains becomes only a scaffolding for fibroplasia (fig. 9). This is true of the fibrin between the wound edge and the fibrin adherent to the curetted stroma.

Epithelial edema persisted over the edematous stroma and the epithelium was pushed up to the surface of the wound as fibroplasia became more intense. The stromal edema was unchanged or slightly less. By this time the wound was completely sealed with a fibroblastic plug equal in the curetted and control eyes as could be determined histologically. There was an intense fibroplasia of the pos-

terior dozen or so lamellae in the areas where Descemet's was absent. This marked fibroplasia was not present where only the endothelium was removed. The two eyes from Experiment I, removed at one week, were larger than the two control globes.

TWO TO FOUR WEEKS

At two weeks stromal edema of the curetted areas was less. Fibrin was of no more importance. There was a generalized increase in stromal fibroblasts in the curetted eyes and especially as mentioned previously in the posterior lamellae over an area of missing Descemet's (fig. 10). Histologically, all wounds were well sealed; both globes from Experiment I were definitely larger than their controls. No further information could be obtained in the one month sections.

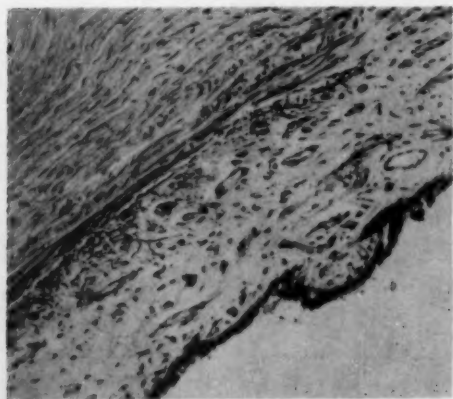


Fig. 10 (McDonald). Experiment II, two weeks postoperative (curetted). Note increased fibroplasia at iris corneal adherence.

DISCUSSION

STROMA

The marked edematous thickening of the stroma of these curetted eyes would be expected from many reports in the literature.^{1, 7, 14} The initial separation of the wound edges immediately after the wound was made, before edema was yet evident, indicates that internal retractive forces in the cornea were liberated by the incision and caused the wound to gape. The gape in

the edematous cornea was greater than in the controls, suggesting that edema does not cause an approximation of the wound as is widely believed. The contrary seems true. Nor does fibrin cause separation of the stromal edges because the gape was greatest in those eyes which had a fibrin deficit. All eyes having a solid fibrin plug had a closer approximation of the stromal edges. This implies that fibrin contraction might be the most important factor in approximating the wound edges.

EPITHELIUM

Others have shown that epithelial migration is resistant to pharmacologic interference. Our results agree with these. In curetted and control eyes alike the epithelial cells migrate down the stromal edges and over the fibrin plug, filling in every crevice and joining it with their neighbors from across the wound. Edema of the epithelium or stroma does not reduce the number of cells which have migrated beyond the wound edge. The distribution of migrated epithelial cells in our histologic specimens could be best explained by the size of the wound gape. There were more migrated cells where the wound edges had greater separation. There was thus more area to cover. This is in agreement with theories of Hartwell¹⁵ and Patterson¹⁶ about skin healing.

FIBRIN

These studies indicate that fibrin adheres to the corneal tissue which is stripped of its normal covering. A firm fixation occurs at the ends of the stromal lamellae, to the posterior stroma devoid of Descemet's and to a lesser extent to Descemet's devoid of endothelium. On the other hand, fibrin adherence to iris does not seem to require defects in its surfaces. Our flat chambers under curetted corneas were a manifestation of the fibrin adhesions. The initial role of fibrin in the production of permanent anterior synechia in these rabbits seems ob-

vious. A possible role of fibrin adherence in human flat chambers cannot be easily disproved. Fibrin deficits in the wound of many curetted eyes suggested mechanical blockage of the fibrin-rich secondary aqueous. We have seen evidence of this in this experiment and in previous experiments.¹⁷ When iris is adherent to the posterior aspects of a central corneal incision, a fibrin plug readily forms over the area where the iris was absent but over the iris adherence the plug was later in appearance and scantier in quality. The iris or lens adhesions produced in these wounds may block the protein-rich aqueous from the wound. The weakened wound produced in humans over an area of partial prolapse may support this contention.

Fibrin adherence seems to invite fibroplasia. Where the fibrin is most intense fibroplasia is greatest. This is true at the wound edge, over Descemet-deficient stroma where fibrin adheres strongly and to a lesser extent over endothelium deficient Descemet's where fibrin adheres less strongly. Hilding¹⁸ gives interesting evidence of this in his through and through corneal sutures. A fibrin conduit covered his anterior chamber sutures and this was later replaced by a fibroblastic conduit.

FIBROBLASTIC PHASE

The eosinophilic polymorphonuclear cells, which rapidly infiltrate the wound edge and fibrin plug, are believed to represent the rabbit equivalent of polymorphonuclear leukocytes in humans. So variable were these in number that we could not relate them to any controllable factor in our experiment. The deficit of stromal cells near the wound edge and over the curetted posterior lamellae seems related to the degree of trauma. That fibrin adhesions and later fibroplasia were greatest in these areas of stromal necrosis seems natural.

At three and four days, fibroblasts were somewhat more numerous in the curetted

eyes, suggesting a greater fibroblastic infiltration in the edematous corneas. As one would expect, fibrinous adhesions which persisted became fibroblastic. That this would lead to secondary glaucoma later was to be expected.

POSSIBLE CLINICAL IMPLICATIONS

Flat chambers in human eyes after a cataract surgery might not always be due to wound leaks or pupil block mechanisms. Investigators might consider the role of adhesive forces between the iris and cornea, as has been demonstrated by fibrin in these rabbit experiments. In eye surgery the meticulous avoidance of trauma to endothelium or Descemet's by instrumentation or excessive irrigation would seem obvious. The possibility that wound leaks and gross evidence of wound weakness frequently associated with flat chambers might sometime be the result rather than the cause of these flat chambers, as suggested by these experimental results, might be considered.

CONCLUSIONS

Endothelial curettment under the conditions of the experiment:

- (1) causes marked stromal edema which does not cause abutment of the wound edges and may even cause their increase separation;
- (2) causes dense fibrinous adhesions of the iris and lens to the curetted cornea resulting in a flat chamber under the curettment, permanent anterior synechias, secondary glaucoma and seems to interfere with the solidarity of the fibrin plug;
- (3) causes epithelial edema but does not interfere with its migration over the wound;
- (4) does not retard and possibly increases fibroblastic activity in the wounded cornea.

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ACKNOWLEDGMENT

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THE EXPERIMENTAL PRODUCTION OF PINE POLLEN GRANULOMATA OF THE IRIS IN HYPERSENSITIZED GUINEA PIGS

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Recent epidemiological studies¹ of a large series of patients with sarcoidosis in the United States, revealed a concentration of cases in the southeastern section of the country, correlating with forest distribution, especially pine forest. This led to investigations as to the possibility of pine pollen being an etiological factor in sarcoidosis.

Cummings et al.² studied the chemical composition of pine pollen, and found it to contain an acid-fast lipid fraction similar to one fraction of mycobacterium tuberculosis and alpha epsilon diamino pimelic acid. These investigators also demonstrated that intracutaneous injections of pine pollen in hypersensitized guinea pigs (using Freund's adjuvant) invoked a granulomatous reaction, which histologically resembled sarcoidosis.

In view of the study of Cummings, experiments were undertaken to produce granulomatous iritis in hypersensitized and non-hypersensitized guinea pigs.

METHODS AND RESULTS

Pigmented guinea pigs (Eldridge Rabbitry) weighing 400-600 gms were used in this study. The pine pollen was from loblolly pines collected in the vicinity of Macon, Ga.

The guinea pigs were divided into 3 groups:

I. Pine pollen injected by means of a 27 gauge needle into one anterior chamber of six non-hypersensitized guinea pigs evoked no clinical reaction. Histologic sections were within normal limits.

II. Pine pollen injected into one anterior chamber of each of six guinea pigs two weeks after sensitization with 0.5 ml of Freund's adjuvant alone (0.4 ml intradermally + 0.1 ml intraperitoneally), caused a reaction after four days consisting of conjunctival injection, ciliary flush, and aqueous flare. This disappeared in an additional four days and histologic section revealed no abnormalities. No reaction at all was noted in the opposite uninjected eye. Injection of saline in one anterior chamber of the control animal, also hypersensitized with Freund's adjuvant alone, resulted in no clinical reaction.

III. Pine pollen injected into one anterior chamber of six guinea pigs two weeks after sensitization with 0.5 ml Freund's adjuvant plus pine pollen (0.4 ml intradermally + 0.1

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Fig. 1 (Coleman). Eye of a guinea pig hypersensitized with Freund's adjuvant and pine pollen 1 week after injection of pine pollen—note iris nodules (×16).

ml intraperitoneally) resulted in the formation of granulomata of the iris after 14-21 days. The reaction varied with the amount of pine pollen injected into the anterior chamber. Although similar to that of group II, instead of disappearing after eight days, the reaction persisted. Grayish raised iris nodules appeared, located in the stroma as well as in the peripupillary area (fig. 1-3). Histologic section revealed these nodules to be composed of epithelioid and giant cells

(fig. 4-6). The lesions resemble those seen in sarcoidosis.

The opposite eyes of these animals served as controls. They were injected with saline and revealed no abnormalities clinically or histologically.

DISCUSSION

Iris granulomata could be produced by the injection of pine pollen into the anterior chamber only in those animals hypersensi-

Fig. 2 (Coleman). Same eye 17 days after injection of pine pollen—note large iris nodules (×10).

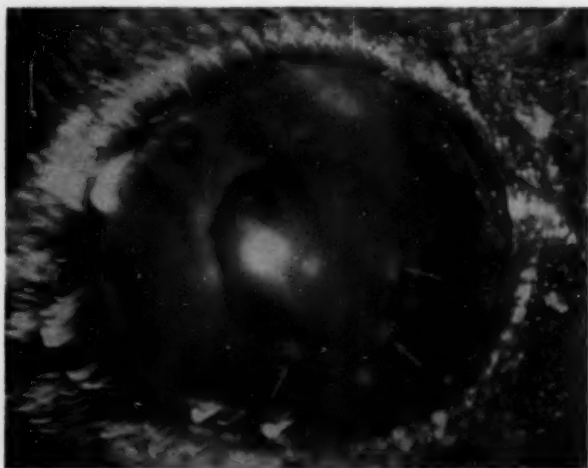
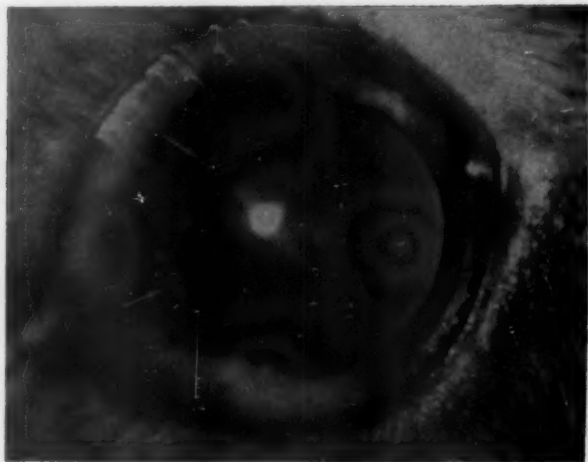


Fig. 3 (Coleman). Eye of another guinea pig hypersensitized with Freund's adjuvant and pine pollen 17 days after injection of pine pollen—note large iris nodules ($\times 10$).



tized with pine pollen and Freund's adjuvant and not with Freund's adjuvant alone, suggesting a direct antigen, antibody reaction of the delayed type. This result is similar to that found by Hudgins.⁸ He prepared an antigen from pine pollen which produced a delayed type reaction in guinea pigs previously sensitized with pine pollen. An attempt to use this antigen as a skin test for the diagnosis of sarcoidosis was unsuccessful. At present, attempts are being made to demonstrate pine pollen and its derivatives in sar-

coid granuloma, and also to demonstrate specific antibody against pine pollen in the sera of patients with sarcoidosis.⁹

Although previous sensitization with pine pollen and not adjuvant alone was required to produce the granulomata in this study, Bullington and Waksman⁴ were able to produce an iridocyclitis in 10 per cent of rats with a single injection of Freund's adjuvant into the hind foot pad. This was associated with arthritis, subcutaneous ear nodules, dermatitis and urethritis. However, the iri-

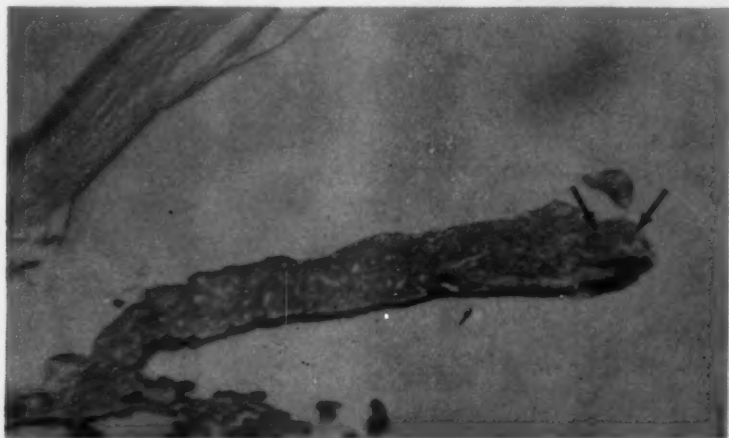


Fig. 4 (Coleman). Histologic section of fig. 1—note nodules in pupillary area of iris (H + E $\times 30$).



Fig. 5 (Coleman). Fig. 4 (H + E $\times 100$)—note nodules composed mainly of epithelioid and giant cells.

docyclitis differed histologically from the nodules described in this report. Bullington and Waksman⁴ found massive infiltration by histiocytes and lymphocytes in the iris and ciliary body, whereas the nodules produced in this study were discrete and composed almost entirely of epithelioid cells. Pine pollen thus may be added to the list of biologic substances capable of inducing epithelioid

cell granulomata. However, further investigations are indicated to confirm or disprove pine pollen as an etiologic factor in sarcoidosis.

SUMMARY

Injection of pine pollen into the anterior chamber of guinea pigs previously sensitized with Freund's adjuvant and pine pollen re-

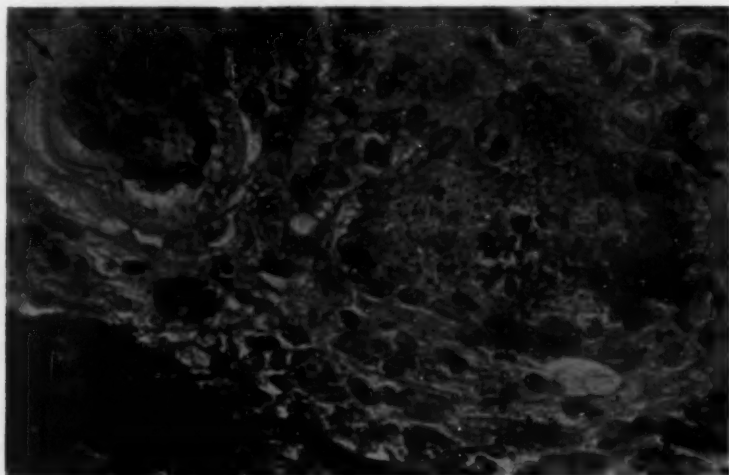


Fig. 6 (Coleman). Fig. 4 (H + E $\times 450$).

sults in granulomata of the iris after 14-21 days.

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THE EFFECT OF CORTICOSTEROID HORMONES ON EXPERIMENTAL HERPES SIMPLEX KERATITIS

A CLINICAL, HISTOPATHOLOGIC, AND HISTOCHEMICAL STUDY

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It is generally agreed that the topical use of corticosteroid hormones has a deleterious effect on herpes simplex. We have shown this effect in previous studies.¹ The experimental disease in rabbits treated with topical corticosteroids was a much more severe disease and had a more prolonged course. This effect was thought by us to be due to an increased multiplication of the virus, because corticosteroids have been shown to have this effect on other viruses.^{2,3} Kilbourne and Horsfall showed that cortisone caused an increased influenza virus multiplication in chicken eggs, and Teodoru and Schwartzman showed the same effect in experimental poliomyelitis in hamsters. Jawetz, Okumoto and Sonne,⁴ however, were not able to show that

corticosteroids increase the amount of herpes simplex virus in rabbit corneas. This suggests that the effect is not due to suppression of antibody formation by the steroid. The only other possibility is that the drugs probably alter the cornea so as to decrease its resistance to the herpes simplex virus. This investigation is designed to study the cellular effects of this virus on corneal tissues as determined by clinical and histologic changes.

MATERIALS AND METHODS

Animals. Black Dutch rabbits weighing approximately two kilograms were used. All of the injections were performed with the eyes anesthetized with one drop of Ophthaine.

Virus. The PH ("O") strain of herpes simplex virus was used. The stock virus was prepared by injecting mouse brain and making a 20 percent mouse brain suspension in skim milk. The preparation was stored at -40°C. The twenty-third mouse brain passage having an LD50 of 10⁻⁸ was used.

Steroids. Prednisolone acetate (Meticor-

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telone acetate^R) 25 mg/ml was used. Two days prior to the virus injection 0.2 ml (5 mg.) of the suspension was injected subconjunctivally and it was repeated every other day for the length of the experiment.

Steroid vehicle control. The vehicle solution of Meticortelone acetate was prepared according to the formula issued with each multidose vial (phenylethyl alcohol 5 mg/ml, benzalkonium chloride 0.1 mg/ml, H₂O C.P.). This vehicle solution was injected subconjunctivally (0.2 ml) into eyes of the control series.

Methods. Thirty rabbits were divided into three groups. Group I) 12 rabbits were given subconjunctival injections of corticosteroids to each eye and two days later were injected with 0.03 ml of the virus suspension PH M.23 beneath Tenon's capsule of each eye; Group II) 12 rabbits were treated in the same manner as Group I, except that the steroid vehicle solution was substituted for the steroid suspension; Group III) control series, six rabbits were injected with the virus and treated with injections of saline regularly every other day until the animals were sacrificed.

Clinical examination of the rabbits were made every other day and the cornea and anterior chamber of the rabbits were studied with a biomicroscope.

Rabbits from each group were sacrificed daily from the fourth to nineteenth postvirus inoculation day. The eyes were immediately fixed in 20 percent formalin solution. After fixation for 24 hours the eyes were cut and corneal specimens were taken for sectioning. The following histologic and histochemical stains were used: Hematoxylin and eosin (H & E), colloidal iron (AMP), Alcian Blue, Giemsa, toluidine blue, periodic acid-Schiff (PAS), and Verhoeff's elastic tissue stain. The stained sections were studied with a light microscope.

RESULTS

Clinical. Clinically the corticosteroid treated animal developed a slight discharge

and redness of the eyes as early as the second day after the virus was inoculated. By the fourth day all these animals developed a moderately severe keratoconjunctivitis. The control animals, on the other hand, developed keratitis about the fourth day but clinically only a few corneal lesions were seen with the biomicroscope and often only in one eye. There was no discharge until the fifth day or later.

The course of the clinical disease was much longer in the corticosteroid treated series. The corneal lesions progressed until the cornea was often completely denuded of the epithelium and the stroma was edematous and hazy. Of the six treated rabbits observed beyond the eleventh postvirus inoculation day, all developed a diffuse epithelial lesion with edema. Three developed shallow ulcers (disciform keratitis). Five of the six rabbits developed uveitis on or about the twelfth day. The control series observed beyond the eleventh postinoculation day developed corneal scarring but all of the corneas were healed by the fourteenth day. Two rabbits developed bilateral uveitis in this group. The clinical picture is summarized in Table I.

Pathological. Hematoxylin and eosin stained sections of the treated and control eyes showed the typical epithelial lesion in the early stages. The epithelial cells bordering the dendritic ulcer showed intranuclear inclusions and viral-type giant cells. The corticosteroid treated eyes showed the presence of inflammatory cells in the epithelial ulcer at an earlier period. This is in keeping with the clinical picture which showed slight discharge in the treated eyes from the second day after virus inoculation. The control eyes showed the presence of inflammatory cells in the epithelial lesion after the seventh day.

The stroma of the cornea also appeared different in the corticosteroid treated eyes. There was edema from the fourth day as contrasted with controls which showed edema on the seventh day. This edema persisted longer in the steroid treated eyes. The

^R Meticortelone acetate furnished by Schering Corporation.

TABLE I

Days Post-inoculation	Steroid	Steroid Vehicle Control	Saline Control
4	Keratoconjunctivitis	Keratitis—slight	Keratitis—slight
5	Keratoconjunctivitis—Moderately severe	Keratoconjunctivitis—Mild	Keratoconjunctivitis—Mild
7-8	Keratoconjunctivitis—Severe	Keratoconjunctivitis—Mild	Keratoconjunctivitis—Mild
10-11	Keratoconjunctivitis—Severe	Keratoconjunctivitis—Mild, healing	Keratoconjunctivitis—almost healed
12-13	Keratoconjunctivitis—Severe Disciform keratitis Uveitis (5/6 eyes)	Cornea healed Uveitis (1/3 eyes)	Eyes healed
14-19	Disciform keratitis Uveitis Severe corneal scarring	Most cases healed 2 cases uveitis	

control eyes showed no edema after the twelfth day. Along with edema there were increased numbers of corneal fibrocytes in both the treated and control series after the twelfth day. The nuclei of these cells often were larger and rounder than normal. The steroid-treated eyes also showed an earlier stromal infiltration of inflammatory cells. There were neutrophils from the fourth day in the treated rabbits but not until the eighth day in the control eyes.

Necrosis of the superficial stroma of the cornea was present in the corticosteroid treated eyes from around the twelfth day. This was not seen in the control eyes. The necrosis is in keeping with the clinical finding of disciform keratitis in the treated rabbits after the eighth day.

Inclusion bodies persisted in diminishing number up to the nineteenth day in treated eyes. In the control eyes none were seen after the twelfth day. Most of the inclusion bodies in the late stages were of the eosinophilic type.

Giant cells were found through the twelfth day after virus inoculation in both series.

Histochemical studies. Corticosteroid treated and control corneas were subjected to histochemical procedures. Under the conditions of the experiment we were not able to determine any significant alteration in the mucopolysaccharide or collagen content

of the cornea. Edema of the stroma caused all of the stains to be lighter in color in both the treated and control series.

DISCUSSION

Experimental herpes simplex keratitis in the rabbit is more severe following treatment with corticosteroid hormone (Prednisolone acetate). The treated eyes develop a more severe keratoconjunctivitis with hyperemia and discharge which has an earlier onset and lasts longer. Histopathological examination of the cornea shows that treated cases develop more extensive epithelial lesions with stromal edema and infiltration of inflammatory cells. Inclusion bodies persist longer in the treated corneas but the viral type giant cells were not seen after the twelfth postinoculation day in both series. The giant cells are formed by the condensation of chromatin into smaller locules, each containing inclusion material. The loculated nuclei continue to grow as the inclusions increase in size. Each compartment assumes the size and shape of an individual nucleus. Therefore, giant cells are found only during the stage that virus is multiplying and their presence probably indicates viral activity in the cornea.

The presence of edema and inflammatory cells in the corneal stroma indicates a more severe keratitis. Necrosis of the superficial

corneal stroma also indicates a more severe reaction. Clinically this is seen in the late stages of disciform keratitis.

The corneal fibrocytes were more numerous and were larger, but no intranuclear inclusions were seen in these cells.

If the deleterious effect of the corticosteroid hormone on herpetic keratitis is due to changes in the corneal ground substance, and acts by decreasing its resistance to spread of the infection, one might expect changes in permeability of the ground substance of the cornea. Two of the functions of the ground substance are thought to be: 1) to act as a mechanical barrier against spread of foreign substance; and 2) to provide a substrate for repair of connective tissue. It is known that corticosteroids decrease connective tissue elements such as fibroblasts and thus prevent normal wound healing. Therefore, histochemical stains and reactions were applied but no change was found. The difference in amount of mucopolysaccharide present may be too small to be detected by these stains. It is possible that the resistance of the cornea is decreased by an epithelial disease of such severity that the epithelium is lost. Smelser⁵ felt that the synthesis of the ground substance is accomplished by the epithelium of the cornea and the connective tissue. He interpreted the role of the epithelium as a source of energy or as a precursor necessary to the synthesis of chondroitin sulfate and/or keratosulfate.

FURTHER RESEARCH

It is certainly evident that the mechanism by which the corticosteroids cause more severe corneal disease in herpetic keratitis remains unanswered. Perhaps electron microscopic examination of tissue sections will reveal whether there is prolongation of survival of the virus. Kilbourne⁶ showed this to be true of influenza B virus in chick embryo cultures. Electron microscopic studies are now under way in our laboratories. Perhaps another approach to the solution of this problem is to carry out enzyme histochemistry, for it is conceivable that the decreased resistance of the cornea is a function of enzymatic action.

SUMMARY

1. Clinical herpes simplex infection of the cornea runs a prolonged course and many of the animals develop a picture of disciform keratitis on prolonged treatment with Prednisolone acetate.
2. Histopathologically the steroid treated rabbits show more extensive epithelial and stromal involvement.
3. Histochemical studies under the conditions of the experiment failed to show any change in the mucopolysaccharide or the collagen of the cornea.

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CHANGES IN THE LENS INDUCED BY MATERNAL HYPERSENSITIVITY IN MICE

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While studying the teratogenic effects of brain antigen on development in mice, we found that injury had occurred to the embryonic lens.

In vitro experiments have demonstrated the presence of substances in the chick embryo capable of combining with adult lens antibodies as early as 35-50 hours incubation.¹⁻³ van Doorenmaalen,⁴ using fluorescein-labeled anti-adult lens serum, found that the marginal zone of the embryonic lens epithelium showed a brilliant fluorescence of the cytoplasm in the 60-hour chick. Certain non-specific embryonic antisera (Ebert,⁵ 1950) and various anti-adult organ sera (Flickinger, Levi, and Smith,⁶ 1955) have been shown to arrest and alter morphogenesis. Injury to the embryonic lens fibers in vivo was described by Burke, Sullivan, Petersen, and Weed⁷ in 1944. They found enlargement and cellular change in the primary fibers of the chick lens following injections of adult lens antiserum.

According to Finlay⁸ and Flickinger et al.,⁶ lens damage to a mammalian embryo by a specific reaction (lens antigen) has not been verified. While the teratogenic effects of brain antigen were reported by Gluecksohn-Walesch⁹ and Barber,¹⁰ no evidence was presented indicating injury to the embryonic lens fibers. We feel that our cataractous results, due to a nonspecific antigenic reaction (hypersensitivity), should be recorded in the literature at this time as its possible correlation with heretofore unexplained

congenital cataracts in the human population should not be overlooked.

MATERIALS AND METHODS

Two types of experiments were devised to test the effects of brain antigen on the development of young in mice. One hundred sixty mature female mice were divided into three groups as follows: Group I, received injections of homologous brain; Group II, received injections of heterologous brain (rabbit); and Group III (controls), received 30 percent Freund's complete adjuvant and 70 per cent normal saline.

Antigen. An emulsion was prepared according to the following formula: 15 percent brain; 30 percent Freund's complete adjuvant; 55 percent normal saline. Only the cortices were used. The brain material was emulsified in a tissue grinder to the consistency of a very thin paste.

Procedure. 0.05 percent intramuscular injections were given in each hind leg three times weekly. The treatment was given in two courses followed by a challenge dose on day-one in pregnancy. The females were allowed to deliver their litters following the first course of treatment, but after the second course, the embryos were examined on the fourteenth and nineteenth day in gestation.

RESULTS

Deaths from anaphylaxis began occurring after the sixth injection in the first course of treatment and 48 animals were lost before this portion of the experiment was ended. Histologic examination of the brains and lungs of these animals revealed thrombi of antigen-antibody precipitates comparable to those described by McKinnon¹¹ for hypersensitivity reactions to be present. Precipitin tests on the remaining females were positive

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for circulating antibodies against brain.

A total of fourteen injections plus a challenge dose were given in the first course of treatments, and six injections followed by a challenge were given in the second course. The challenge dose consisted of a 0.025 cc. injection in each hind leg given one week following the course of treatments.

Only eight females in Group I (mouse brain antigen) survived the second course of treatment and produced seventy embryos of which forty-six had lens defects. Eleven females in Group II (rabbit brain antigen) survived the second course and produced ninety-seven embryos of which sixty had defective lenses.

Lens. In the mouse the lens vesicle separates from the surface ectoderm on the eleventh day in gestation, and by the twelfth day, the primary fibers are beginning to elongate. There is no cytological evidence of injury to the lens at this stage in embryos from treated mothers (fig. 1). By the fourteenth day the primary fibers have reached the anterior wall of the lens vesicle and obliterated its lumen. The condition of the lens



Fig. 1 (Barber, Willis and Afeman). Section through the head of a mouse embryo from a sensitized mother. Eleventh day in gestation. The lens vesicle is still attached to the surface ectoderm. There is no evidence of injury to the lens at this time.

of an embryo from a sensitized mother is shown in Figure 2. Very fine vacuoles and droplets can be seen in the primary fibers. Figures 3, 4, 5, and 6 show the condition of the lens on the nineteenth day in gestation at which time liquefaction and cystoid degenerative changes have occurred in the center of the lens. A few layers of secondary fibers are formed at this stage in development but appear to be undamaged.

There were no essential differences found in the effects of homologous and heterologous brain emulsions.

Grossly the lenses of these embryos appear densely opaque on the nineteenth day (fig. 8); the gestation period is twenty days. No lens defects were found in the controls.

DISCUSSION

The cytotoxic effects of a number of anti-organ sera have been studied, including brain, lens, placenta, heart, kidney, lung, and spleen.^{1,2,12} Lewis,¹³ Morgan,¹⁴ Kabat, Wolf, and Bezer,¹⁵ Morrison,¹⁶ and Olitsky and Tal¹⁷ produced encephalomyelitis rapidly and reproducibly in several types of experimental animals by injections of brain extracts. Contrary to their experience, we found no evidence of encephalomyelitis in any of our animals, either in the treated mothers or in their embryos.

The teratogenic effects of maternal treatment with adult brain emulsions were reported by Gluecksohn-Waelsch⁹ and Barber¹⁰ in mice. Further study of these data, however, seems to indicate that the anomalous development may have been complicated to a marked degree by trauma and intra-uterine death. Larger doses of antigen were given and in a shorter period of time in those experiments than in the ones reported here.

According to Boyd¹ the antibody response reaches a maximum intensity seven days following the challenging dose and maintains this intensity through the fourteenth day in most animals. The rise in antibody response appears to correlate with the peak incidence of anaphylactoid deaths in our mice, and to

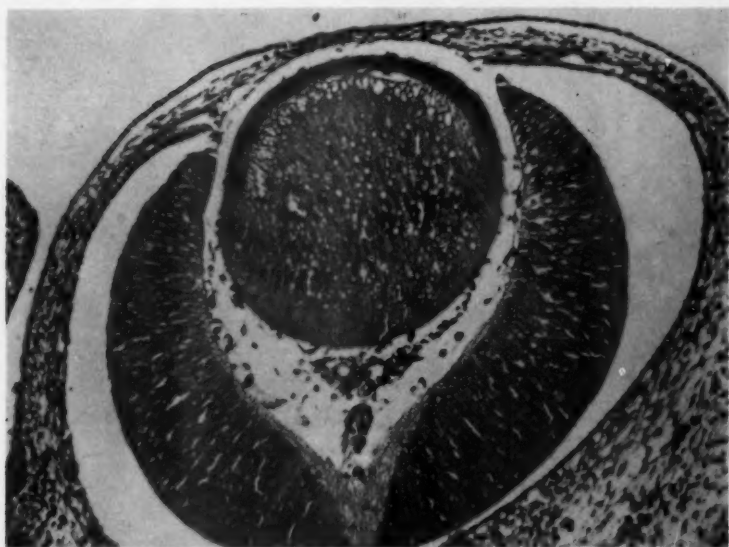


Fig. 2 (Barber, Willis and Afeman). Section through the eye of an embryo from a sensitized mother. Fourteenth day in gestation. The primary fibers have reached the anterior wall of the lens vesicle. Numerous small cystoid spaces are present in the primary fibers.

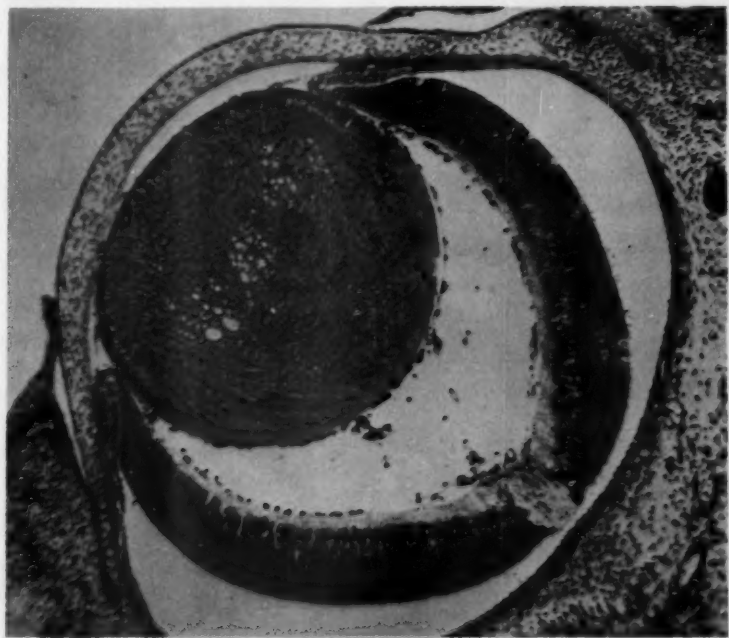


Fig. 3 (Barber, Willis and Afeman). Section through the eye of an embryo whose mother was sensitized to brain. Nineteenth day in gestation. Several layers of secondary lens fibers are formed and degenerative changes can be seen in the primary fibers.

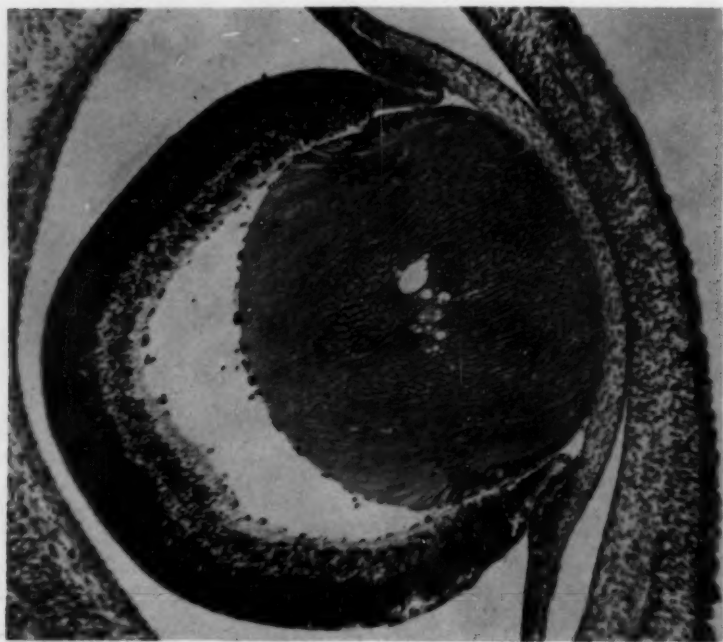


Fig. 4 (Barber, Willis and Afeman). An eye of an embryo on the nineteenth day in gestation. Cystoid degenerative changes are limited to the center of the lens.

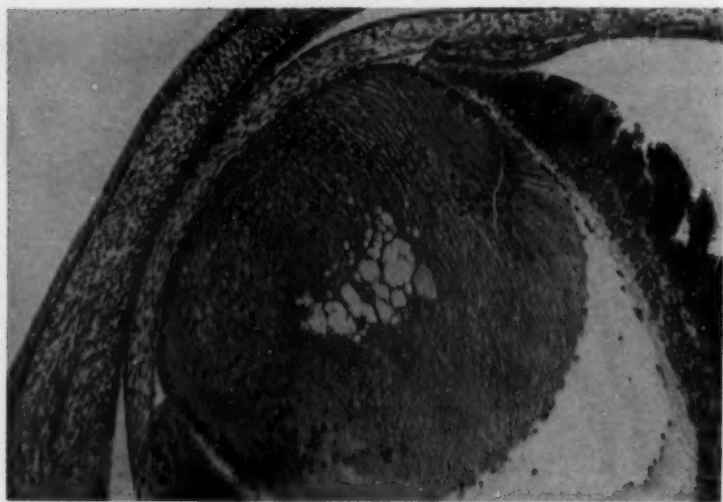


Fig. 5 (Barber, Willis and Afeman). Lens of embryo on nineteenth day in gestation. Numerous large fluid-filled spaces are located in the center of the lens.

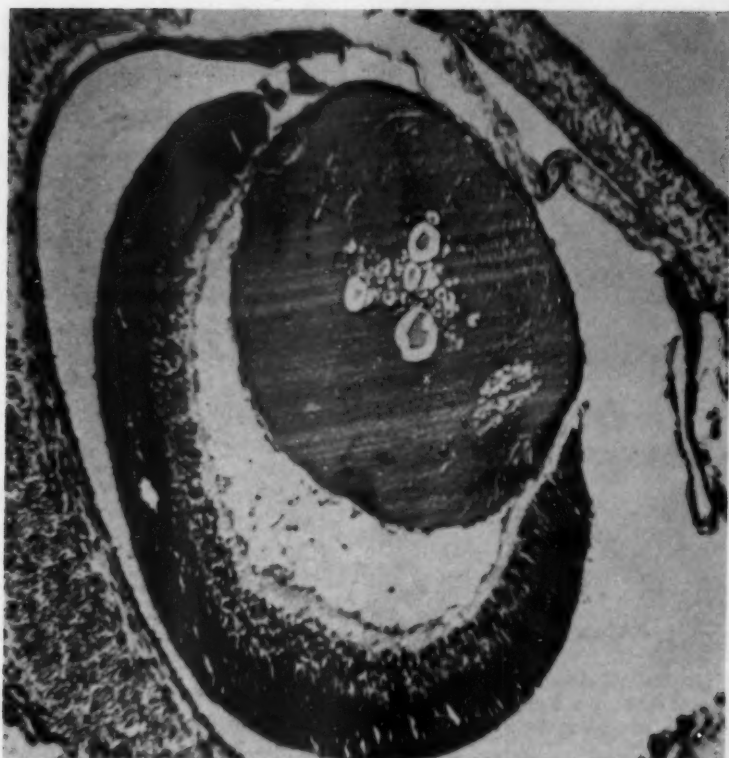


Fig. 6 (Barber, Willis and Afeman). Lens of embryo from treated mother on nineteenth day in gestation. The large spaces contain an amorphous pink-staining material.

parallel the time of appearance of lens defects following the second course of treatment. At present we have no explanation for the fact that toxic effects to the lens followed the second course of treatment but did not occur after the first course. The precipitation tests were positive at the end of the first series of injections and approximately the same number of anaphylactoid deaths occurred. Pregnancy does not seem to be the answer since a few of the mice failed to become pregnant after the first series of injections and did produce embryos with lens defects following the second course of treatments.

By far the greatest amount of information pertaining to the antibody response of the lens has accumulated from studies on the

chick embryo and from tissue cultures of embryos of various other species. Burke⁷ et al., injected adult lens antiserum into the chick embryo and produced enlargement and cellular change in the lens fibers. They felt that, after the lens begins to assume the adult texture, it does not react to a specific antiserum and may not be absolutely organ specific.

The nonspecific nature of lens antiserum was described by Finlay⁸ who injected anti-lens serum into pregnant rats and mice and found no toxic effects on the embryos. Flickinger et al.,⁹ confirmed these findings by injecting emulsions of cattle lens into pregnant rats.

The cataractous changes in the lenses of embryos from sensitized mothers in our ex-



Fig. 7 (Barber, Willis and Afeman). Mouse embryo from sensitized mother. Nineteenth day in gestation. The dense opacity of the lens can be seen through the eyelids.

periments seem to result from a nonspecific antibody response. These changes resemble

very closely the cytologic changes found in the human embryonic lens following maternal rubella described by Barber.¹⁸

SUMMARY

The effects of a nonspecific antigenic reaction (hypersensitivity) on the embryonic lens in mice are described.

Female mice were sensitized by injections of homologous and heterologous (rabbit) brain emulsions and given a challenging dose on the first day of gestation. Histologic sections of the embryonic lens show the primary fibers to contain very small vacuoles and droplets. By the nineteenth day, large vacuoles and cystoid degenerative changes are found in the center of the lens. The secondary fibers of the lens do not appear to be affected.

The maximum response of antibody occurs seven to fourteen days following a challenging dose of antigen and the injury to the embryonic lens in these experiments appears to parallel in time and to result from this optimum response of antibody.

There appears to be no essential difference found in the effects of homologous and heterologous brain emulsions.

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STUDIES ON THE CORNEAL ENDOTHELIUM OF THE RABBIT

I. CELL DIVISION AND GROWTH

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The morphology of the corneal endothelium cannot be studied adequately by routine histologic procedures. Flat sectioning and flat mount techniques were used, therefore, by many workers.¹⁻¹³ As a rule only pieces of the endothelium could be secured for microscopic examination. Nagano¹⁶ greatly improved on the method of obtaining flat mounts of this cell layer, but limited his study to regeneration processes after injury. Recently, Mills and Donn¹⁷ investigated the uptake of tritiated thymidine by endothelial cells using a flat mount technique. With a procedure developed in our laboratory,¹⁸ complete flat mounts of the endothelium attached only to Descemet's membrane were obtained regularly from the rabbit's cornea. It was possible, therefore, to study such unsolved problems as cell renewal and the relation of cell proliferation to the growth of the endothelium. Diurnal changes in mitotic activity and the role mitosis and amitosis may

play in growth and replacement could be investigated also.

MATERIAL AND METHODS

A cross-bred strain of New Zealand white rabbits supplied by the NIH Animal Production Section was used exclusively. The animals were killed at the age of two weeks, one month, two to three months, one year, or two to three years. In all experiments, such external conditions as housing, diet and room temperature were kept as constant as possible.

Preparations from both eyes of 60 animals were examined in the present study. The endothelium was fixed while the animals were alive, but under nembutal anesthesia, except in the case of the two-week-old rabbits which were decapitated before fixation. The fixative (one part glacial acetic acid in three parts 95 percent ethanol) was injected into the anterior chamber, using a modified Fuchs syringe with a 27 gauge needle, inserted either at the limbus or behind the iris to enter the anterior chamber through the pupil. Following enucleation, the eyes were bisected behind the ora serrata. Vitreous, lens and the radially incised ciliary body and iris were removed without touching the cor-

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† Dedicated to Professor Doctor Arnold Pillat in old friendship.

neal endothelium. The isolated cornea with a rim of sclera surrounding it was placed in the fixative for 24 hours and transferred to 70 percent ethanol for at least the same length of time. In the staining procedure, the preparation was kept for 30 minutes in distilled water, then treated for eight minutes with 1N HCL maintained at 60° in a water bath, and finally placed in Schiff's reagent for at least 30 minutes. The stained cornea was dissected in a small dish filled with distilled water under 20 × magnification. With the preparation lightly supported by the blade of a blunt curved forceps, one tip of a fine pointed forceps (Dumont #5) was inserted under Descemet's membrane in the darkly stained area of the cornea-scleral meshwork. Descemet's membrane was severed from the scleral spur along its entire circumference by blunt dissection and then peeled completely from the stroma. This procedure was greatly facilitated when the separation was started in the proper plane between the two layers. The isolated membrane with endothelium was lifted out of the water on a strip of heavy cellophane, the stroma side facing the strip, and floated onto a drop of water on a cover slip with the endothelium down. Radial incisions were made with a razor blade to allow flattening of the preparation. Folds and wrinkles were eliminated by brushing with a strip of wet filter paper or by traction with the fine forceps; remnants of the stromal fibers could also be removed at this time. Drying of the endothelium during stretching and flattening had to be avoided since it proved to cause considerable shrinkage of the cells. The preparation was finally mounted in 3 percent gelatin and a weight placed on the coverslip to prevent the entrance of too many air bubbles.

The microscopic examination was carried out within a few days, since sometimes bleaching of the stain affected the reliability of the mitotic counts. The entire cell population was systematically scanned under 370 × magnification and differential counts were made in all instances. Early prophases

and reconstruction phases were not included. About one hour is required for the microscopic examination of a normal endothelium mount.

For the determination of the mitotic index, the size of the cell population was calculated according to the method used for the lens epithelium.¹⁹ The slides were projected on a paper screen at a known magnification, the outlines of the mounts traced and the areas measured with a planimeter. The nuclei in 20 microscopic fields, .007 sq. mm. in area, were counted and the counts averaged. From these values and the measured area of the whole preparation, the cell population was estimated in four mounts from each age group.

Diurnal variations of mitotic activity were determined on littermates of two, four, and 12-weeks-old rabbits. One animal of a pair from the same litter was killed between 7:00 and 7:30 A.M. and the other was killed between 4:00 and 4:30 P.M. In four experiments, mitotic activity in the endothelium was determined together with that in the lens epithelium of the same eye and diurnal changes in the activity of both tissues were compared. The technique used for fixation of the endothelium could not be employed to prepare the lens epithelium mounts in these experiments because cells remained attached to the cortex. By necessity, then, the method for fixing the lens epithelium was adopted although it produced shrinkage of the endothelial cells and resulted in moderately inaccurate counts. Prophases and frontal views of metaphases could easily be overlooked under these conditions.

The experiments were limited to rabbits. Attempts to apply the technique to the cornea of rats, cats, guinea pigs and Rhesus monkeys have been unsuccessful so far because Descemet's membrane could not be separated from the stroma in these species.

RESULTS

The flat mounts obtained by this technique were generally good, particularly in young

adult animals. Dehiscences in the endothelium mosaic produced by the extreme tissue volume changes which occurred during the staining procedure did not affect the dependability of the counts. Rubbing off of cells during the spreading of the preparation, residual stromal fibers attached to the mount, and the fine wrinkling of the rigid Descemet's membrane common in old animals, also did not influence essentially the results. Mounts from two week old animals were often incomplete, however, so that the total mitotic count had to be corrected by adding an estimate of the number of mitoses in the missing area. Counting all dividing cells in the entire population is of advantage as mitoses are not uniformly distributed throughout the zone where they occur. None of the usual methods of counting dividing cells in sections can take care of this fact.

The endothelial cells of the young adult rabbit in stained preparations had a diameter of about 15-20 μ and were polygonal in shape. Often the cell margins could not be seen and the presence of interdigitations or of cement substance could not be studied. The leptochromatic nucleoplasm contained one or two nucleoli. The nuclei in younger

animals stained well with Schiff's reagent, but in old animals only faintly. The cell nuclei in the young animals were predominantly oval and later became elongated and kidney-shaped, measuring $8 \times 12 \mu$ (fig. 1). In old animals, many nuclei assumed bizarre dumbbell or crescent shapes (fig. 2). The long axis varied from 18-24 μ , the short axis remained approximately 8 μ . Binucleated cells were often seen in preparations from old animals. In such cells the two nuclei usually were of similar size, but occasionally a small round nucleus and a large crescent shaped one were present in the same cell. Degenerative changes such as pyknosis, extrusion of clumped densely stained chromatin from the nucleus, or vacuolation of the cytoplasm were not observed in the endothelium of the two to three-year-old rabbits.

In the course of preparing the mounts, round or irregularly shaped Feulgen positive dots were noted on the endothelial side of the stained cornea. They were scattered irregularly, but as a rule were located in a peripheral area. When the elastic membrane was peeled from the stroma, these structures offered a very slight resistance and sometimes tended to stick to the stroma. In the mounted

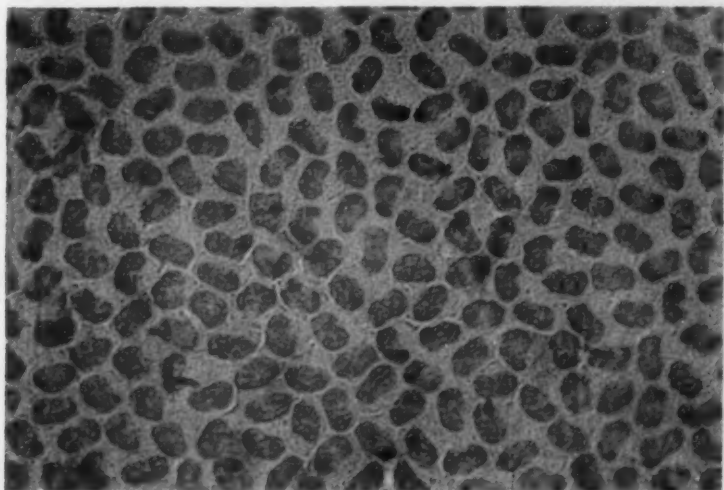


Fig. 1 (von Saltmann, Caravaggio and Grimes). Endothelium mosaic in eight-weeks-old rabbit; $\times 600$.

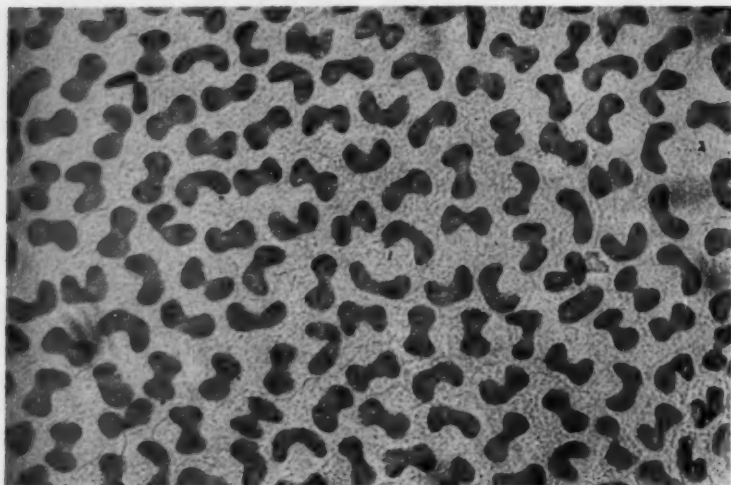


Fig. 2 (von Sallmann, Caravaggio and Grimes). Endothelium mosaic in two and one-half-years-old rabbit; $\times 550$.

preparation, the dots were seen to be composed of small cells with deeply stained nuclei, which did not have the appearance of nuclei of either endothelial or stromal cells. These formations usually exhibited stress lines as shown in Figure 3. Twelve to twenty-four of such cell aggregations could

be counted in a single preparation. In the youngest animals examined, staining of the nuclei was fainter and the packing density of the cells was less marked than in older animals. To determine the location of these cells, small portions containing the darkly stained knobs were cut out from the Feulgen

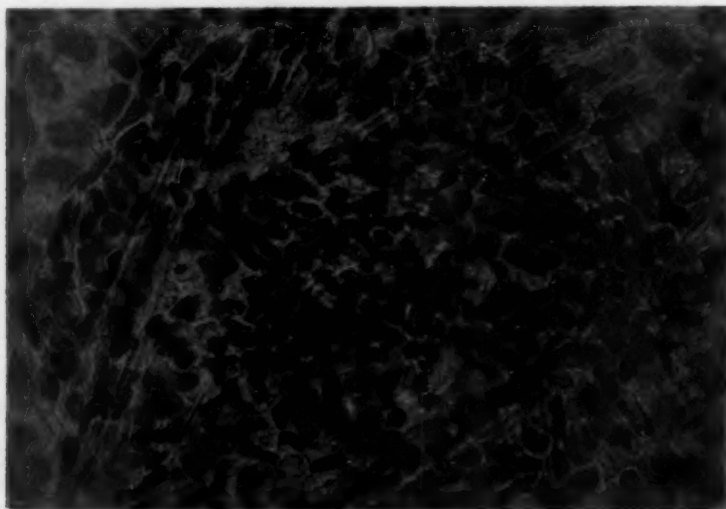
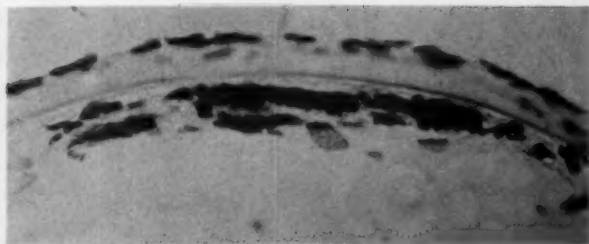


Fig. 3 (von Sallmann, Caravaggio and Grimes). Subendothelial button in twelve-weeks-old rabbit with "stress lines"; $\times 600$.

Fig. 4 (von Sallmann, Caravaggio and Grimes). Histologic section through endothelium, Descemet's membrane and subendothelial button $\times 400$.



processed corneas, imbedded in paraffin and cross-sectioned. The cells were located between Descemet's membrane and the stroma (fig. 4), but their origin could not be established. They did not show mitotic activity, whereas overlying endothelial cells often did (fig. 5) contain dividing nuclei. These peculiar cell formations might serve to increase the cohesion between Descemet's membrane and the corneal stroma in the rabbit.

In the normal endothelium of growing rabbits, mitoses were observed predominantly in a peripheral ring zone of about 1 to $1\frac{1}{2}$ millimeters width (figs. 6 and 7). The dividing cells were not equally distributed in this area but were relatively numerous at some sites and sparse at others. These cells lay usually in the plane of the

neighboring cells; not infrequently, however, they appeared displaced in the direction of Descemet's membrane so that it was necessary to change focus with the high dry objective. Mitoses were seen also in the portion of the corneoscleral meshwork which adhered to Descemet's membrane (fig. 8); here overlapping structures prevented dependable counting. The phase counts showed the usual relationship of 1:2:1 for prophase, metaphase and telophase.

The number of dividing cells was first determined in 8 to 12 week-old animals killed in the afternoon. The mitotic counts varied considerably from animal to animal, but the number of mitoses in the right and left eyes of individual rabbits was usually close (table 1). The average number of mitotic figures

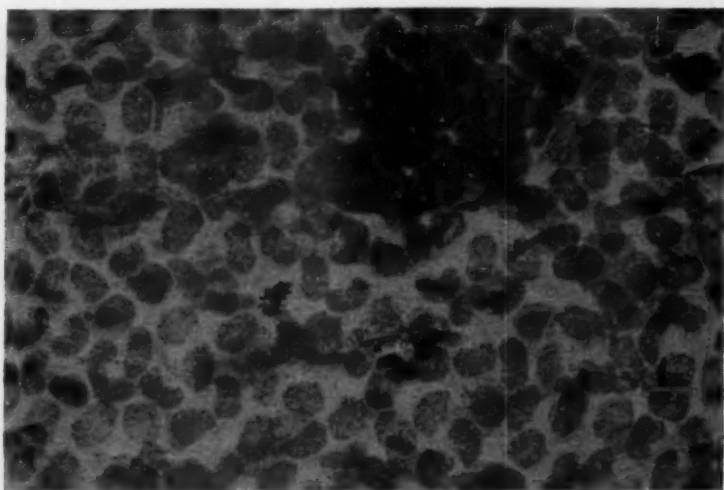


Fig. 5 (von Sallmann, Caravaggio and Grimes). Subendothelial button with mitotic figures in periphery of cornea in a four-week-old rabbit; $\times 600$.

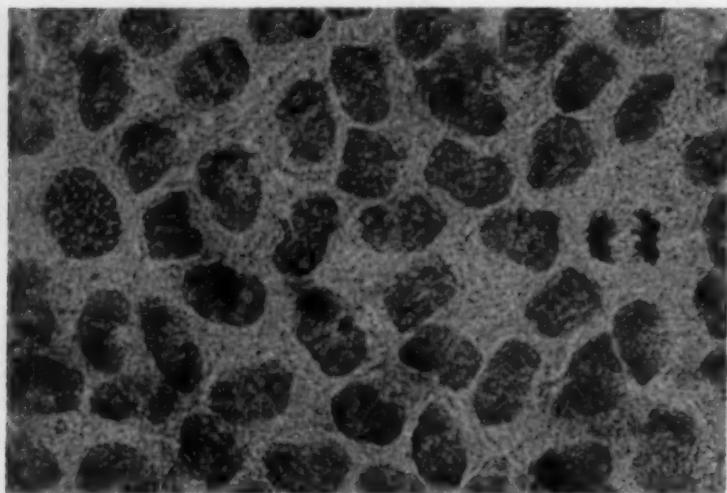


Fig. 6 (von Sallmann, Caravaggio and Grimes). Prophase and telophase in endothelium of eight-weeks-old rabbit; $\times 700$.

seen in a group of eleven animals was 44.3 per preparation. When rabbits between one and two and one-half years of age were examined, very few mitotic figures were noted. In three of the six rabbits studied, no mitoses were present in the endothelium. The remaining three animals had counts between one and three (fig. 9).

The total number of cells in the endothelium of the different age groups was determined in order to calculate the mitotic index (table 2). The nuclei were counted in microscopic fields of known area in both the peripheral and central zones, since there appeared to be a difference in the density of cell population in these two sites. Prepara-

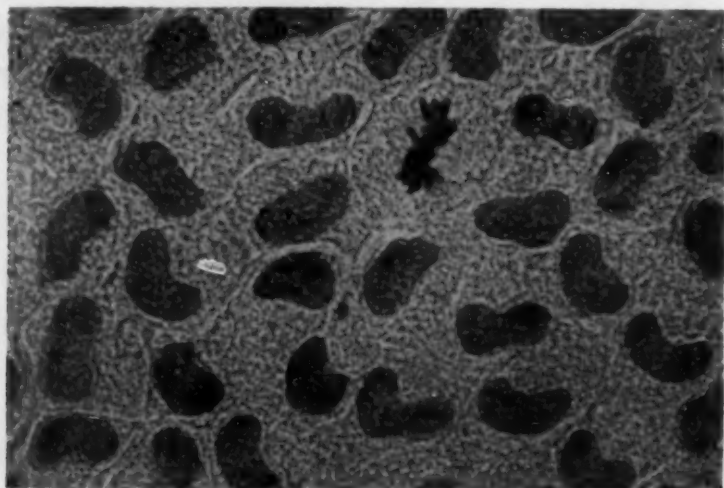


Fig. 7 (von Sallmann, Caravaggio and Grimes). Metaphase in the same preparation as 6; $\times 700$.

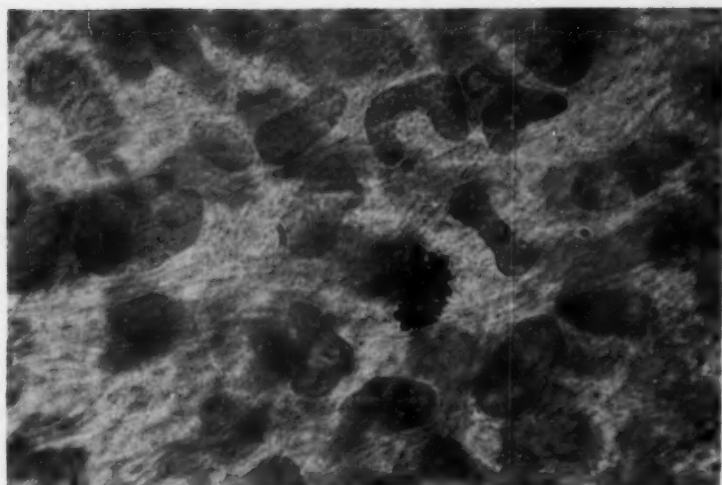


Fig. 8 (von Sallmann, Caravaggio and Grimes). Dividing figure in corneoscleral trabecular area (eight-weeks-old rabbit); $\times 700$.

tions from old animals consistently showed a smaller number of nuclei per unit area in the periphery than in the central portion. An average of peripheral and central counts was used to calculate the number of cells per preparation. A population of 460,000 cells was estimated for the two-week-old animals. This number increased in eight-week-old animals, by less than one-third, to 600,000. The size of the endothelium preparation in this interval, however, more than doubled. This relatively greater increment of area as compared to population is accomplished by spreading of the cells as can be seen in the decreasing number of nuclei counted per unit area. From eight weeks to 26 months, the discrepancy between area increase and change in cell population becomes even more exaggerated. There is a 70 percent enlargement of area while the number of cells increases by only 3 percent.

When the mitotic index is calculated on the basis of these figures and an average of morning and afternoon counts the following values are obtained: 10.4/100,000 for two-week-old animals, 5.7/100,000 at four weeks, and 3.4/100,000 at eight to ten weeks. There

is actually a greater fall in mitotic activity than is apparent from the counts alone. In old animals, the mitotic index is very small, approximately 0.1/100,000.

Considering what is known of mitosis in the corneal and lens epithelium, it was apparent that these counts did not provide sufficient information on the overall mitotic activity of the tissue. It has been reported that over a twenty-four hour period, the number

TABLE I
MITOTIC ACTIVITY IN CORNEAL ENDOTHELIUM OF
8-12 WEEK-OLD RABBITS
(Killed in the Afternoon)

Age (weeks)	Weight in kg.	Number of Mitoses	
		O.D.	O.S.
8	1.8	63	63
8	1.8	20	25
8	1.7	32	34
8	?	38	37
8	1.8	lost	49
8	2.1	27	31
8	2.1	33	42
10-12	2.3	27	18
10-12	2.3	42	50
10	2.0	54	54
10	2.2	61	60
10	1.9	52	49
10	1.9	55	57
		Avg. 42	Avg. 43

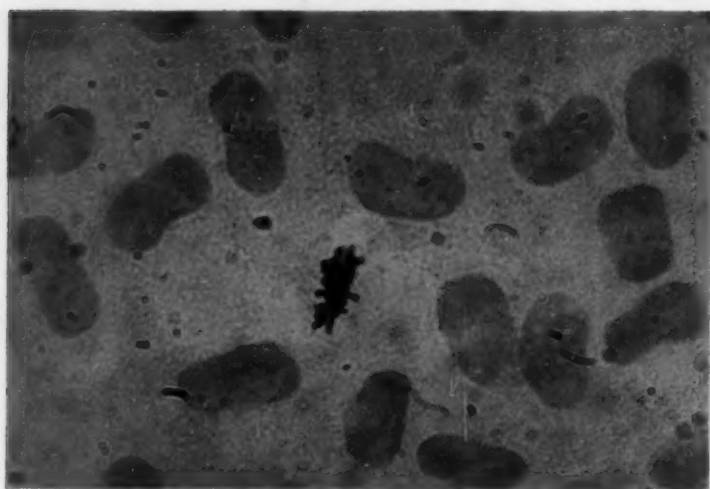


Fig. 9 (von Sallmann, Caravaggio and Grimes). Metaphase in endothelium of two and one-half-years-old rabbit; $\times 700$.

of mitoses observed in the corneal epithelium in mice reached a maximum at 8:00 A.M. and a minimum at 8:00 P.M.²⁰ Studies on diurnal changes were undertaken, therefore, and the hours of 7:00-7:30 A.M. and 4:00-

4:30 P.M. were selected for comparison. Rabbits varying in age from two weeks to ten weeks were used in littermate pairs since in a few experiments, it had been observed that the counts from littermates, killed at the

TABLE II
CELL POPULATION OF THE ENDOTHELIUM IN DIFFERENT AGE GROUPS

Age	Area in sq. mm.	Counts		Population
		Peripheral	Central	
2 wks.	46.7	70	60	440,000
	50.9	66	69	491,000
	52.0	64	57	446,000
	50.0	65	62	457,000
				Avg. 458,000
4 wks.	87.4	37	39	474,000
	82.2	39	53	539,000
	87.4	47	49	586,000
	82.9	44	45	522,000
				Avg. 525,000
8 wks.	126.5	23	34	505,000
	121.6	30	34	558,000
	115.7	34	46	658,000
	122.2	38	39	684,000
				Avg. 601,000
13 mos.	197.0	18	25	620,000
26 mos.	197.0	19	25	620,000
	202.0	19	24	635,000

TABLE III

DIFFERENCES OF MITOTIC ACTIVITY IN THE CORNEAL
ENDOTHELIUM OF LITTERMATES (A AND B)
KILLED IN THE AFTERNOON

Age	A		B	
	O.D.	O.S.	O.D.	O.S.
4 wks.	25	33	40	40
8-10 wks.	61	60	54	54
	52	49	55	57
	31	27	33	42
	Avg. 47		Avg. 49	

same time, were in close agreement (table 3).

In all age groups, the morning counts exceeded by far those of the afternoon (table 4). In ten to twelve weeks old rabbits, mitotic activity was approximately 7.3 times greater in the early morning. The ratio of morning to afternoon counts of the four weeks group was 5.3 and of the two weeks animals was 3.9. A considerable drop from the 7:00-7:30 peak of mitotic activity was observed when rabbits were killed slightly

TABLE IV

DIURNAL CHANGES IN THE MITOTIC ACTIVITY IN
THE CORNEAL ENDOTHELIUM OF RAB-
BITS IN DIFFERENT AGE GROUPS*

Age	7-7:30 a.m. Average OU	4-4:30 p.m. Average OU	Ratio
			Morning Afternoon
2 wks.	205	51	4.0
	239	57	4.2
	149	51	2.9
	210	46	4.6
	201	51	3.9
4 wks.	106	19	5.6
	255	49	5.2
	95	17	5.6
	166	35	4.7
	156	30	5.3
8-10 wks.	87	14	6.2
	274	49	5.6
	99	18	5.5
	132	11	12.0
	148	18	7.3

* In all cases, comparison of morning to afternoon counts was made between 2 animals from the same litter.

later. An average count of 55 was obtained from four rabbits killed between 7:30 and 8:30 in comparison to 148 for the same number of animals killed between 7:00 and 7:30.

Diurnal variation of mitotic activity in the corneal endothelium was compared to that in the lens epithelium of the same eye in four weeks and ten to twelve weeks-old rabbits (table 5). In the four experiments, the number of dividing cells in the lens epithelium was higher in the morning than in the afternoon, corresponding to the variation in endothelial mitotic activity. The magnitude of the change, however, was much less in the lens. The average ratio of morning to afternoon counts was 1.3 in four weeks animals and 1.5 in ten to twelve weeks animals. These figures appear to be in disagreement with those reported in a previous study of the lens epithelium where the mitotic activity was found to be slightly higher in the afternoon.¹⁰ The results of the present study cannot be compared with the earlier work because the counts were made, not in the early morning, but in the forenoon.

DISCUSSION

It is commonly believed that the cells of the normal corneal endothelium do not divide by mitosis except shortly after birth. This opinion is based primarily on observations published in 1900 by Ballowitz^{4,5,6} who studied the endothelium of cats. The alleged absence of mitoses in this cell layer of the growing cornea suggested to Lauber (1936), as it did to Ballowitz, that the cellular units have to flatten out to provide for a continuous cover of the expanding surface of Descemet's membrane in post-natal life. As no evidence for mitotic activity of the endothelium was available, Cogan¹¹ (1951) concluded that "the method and rate of growth of the endothelium is wholly obscure" and Thomas²² (1955) restated this view verbatim in his book on the cornea. The results of more recent studies of Binder and Binder¹⁴ on the rabbit seemed to confirm the impression that mito-

TABLE V
COMPARISON OF DIURNAL CHANGES IN MITOTIC ACTIVITY OF CORNEAL ENDOTHELIUM AND LENS
EPITHELIUM OF THE SAME EYE*

Age	Cornea			Lens		
	7-7:30 a.m.	4-4:30 p.m.	Ratio	7-7:30 a.m.	4-4:30 p.m.	Ratio
			Morning Afternoon			Morning Afternoon
4 wks.	250	98	2.6	218	172	1.3
	90	18	6.9	258	188	1.4
			Avg. 4.8			Avg. 1.4
8-10 wks.	284	50	5.7	330	179	1.8
	97	15	6.4	288	239	1.2
			Avg. 6.1			Avg. 1.5

* Comparison of morning to afternoon counts was made between 2 animals of the same litter.

sis does not exist under normal conditions. These authors inferred that regeneration in the normal endothelium takes place solely by amitosis. On the basis of the results reported in this study the conclusion that no mitosis occurs in the endothelium of the growing eye must be refuted.

In the rabbit, corneal growth is accompanied by mitosis in the endothelium, predominantly in the peripheral portion. The mitotic index of young animals decreases as they mature and approaches zero when growth subsides. Occasionally a few dividing cells are found in rabbits two to three years of age. A minimal loss of endothelial cells in old animals may be replaced by newly formed cells. Mills and Donn¹⁷ did not observe the incorporation of H³ thymidine by nuclei of endothelial cells in adult animals. On the assumption that in their experiments total flat mounts were used, the results are not in full agreement with those obtained in our study by a careful and systematic search for dividing cells in the whole population.

The extent of cellular proliferation during growth deduced from mitotic counts falls short by far from the value which could account for the increased surface of the cornea. As shown by area measurements and cell counts, spreading of the cells pro-

vide a continuous lining for the growing Descemet's membrane. An increase in cell size with wider spacing of the nuclei is evident in photographs of young and old animals taken at the same magnification and is concluded on the basis of the nuclear counts in known microscopic fields in the various age groups. This spreading may be accomplished by either flattening of the cells or enlargement of the cell volume. Such a process was postulated by Ballowitz, Lauber and others as the only means of endothelial growth on the incorrect assumption that mitosis does not occur in the growing animal. The techniques employed in this study allows correlation of the parts played by cell division and cell spreading in expansion of the endothelium. This cell layer is, in fact, uniquely suited for the study of such a relationship.

The morphology of the cells and the cell nuclei undergo considerable change as the rabbit ages. The long axis of the nuclei in old animals is almost double that of the nuclei in younger animals, while the short axis remains approximately the same. These nuclear changes are similar to, although not as marked as, those described by Ballowitz in the cat and sheep. In many endothelial cells of old rabbits, two nuclei are observed. Binucleated cells occur also in younger age

groups, but less frequently. Claims have been made in the past that amitosis participates in the regeneration of the endothelium after injury^{7, 13, 14, 16} and Binder and Binder¹⁴ postulated it as the way by which the normal endothelium grows. All proponents of this hypothesis arrived at their concept by studying fixed preparations. It is generally accepted now that conclusions based on such material are not sound. Direct nuclear division may take place in the endothelial cells without subsequent division of the cytoplasm. In the more accepted view, this process is considered to be a form of nuclear fragmentation and not an indication of cellular proliferation.²⁴ To whichever opinion one adheres, the cause and the functional significance of direct nuclear division, in case it exists, is completely unknown.²⁵ The results of examination of whole mounts reported here make it superfluous to resort to amitotic cell division as a means of growth of the endothelium. Although mitotic activity sharply decreases in the maturing animal, the number of newly formed cells also declines and compensatory spreading of the cells becomes predominant in adapting the endothelial area to that of the growing cornea.

According to the classification of tissues by Leblond and Walker²³ the corneal endothelium is to be considered a non-renewal population, in which the number of cells of the non-growing animal remains constant without mitosis. It shares this characteristic with the vascular endothelium and the peritoneal serosa. The cornea, then, is covered by a renewal tissue on its outer surface which is exposed to irritants, and is lined on its inner surface by a cell layer bathed in a fluid of constant composition which is not subjected to irritation. In both cell layers, diurnal changes in mitotic activity are observed. Vasama and Vasama²⁰ have extensively studied in histological sections, the diurnal cycle in the corneal epithelium of the mouse. Their results resemble those of Blumenfeld^{26, 27} and Bullough^{28, 29}

obtained in the epidermis of the rat and mouse. In these tissues and species, the rhythmical changes in cell division show a peak in the morning and a marked depression in the afternoon. The endothelium of the rabbit appears to follow these tissues in their diurnal cycle.

In the lens epithelium, studied previously,¹⁹ data are not sufficient to compare the results with those obtained in the corneal endothelium. It is obvious, however, that the swing of the pendulum of the biological clock is much greater in the corneal endothelium than in the lens of the rabbit.

SUMMARY

1. A method of preparing whole flat mounts of the rabbit corneal endothelium was developed which allowed reliable mitotic counts in the entire cell population.

2. Mitotic cell division regularly occurs in the growing animal mostly in the peripheral zone of the cornea. The mitotic index decreases with age and approaches a value of zero in the full grown animal. The endothelium is considered a non-renewal tissue, in which mitosis practically subsides when growth stops.

3. In addition to cell division, flattening of the cells and possible gains in cell volume are mechanisms by which the endothelium increases in area. The latter processes play a more important part in the growth of the tissue than cell division.

4. Diurnal variations of mitotic activity in the endothelium are marked. The ratio of early morning to afternoon counts in the young animal range from 3.9 to 7.3. These changes resemble the rhythmic variation observed in renewal tissues such as the corneal epithelium and the epidermis of the mouse.

5. Circumscribed aggregations of small cells with darkly stained nuclei located between stroma and Descemet's membrane are thought to strengthen the cohesion between the two layers.

Bethesda, Maryland.

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DISCUSSION

DR. FREDERICK W. STOCKER (Durham, North Carolina): Dr. McDonald has made an admirably thorough study on how a defect of the endothelium adjacent to a corneal incision may affect the healing of a wound. He has confirmed some known facts and added new information. Ever since the classic experiments by Leber in 1873 (*Graefe's Arch. f. Ophth.*, 19:Abt. 2: 87-185) it has been known that the endothelium of the cornea prevents the aqueous from penetrating into the corneal stroma and that a deficient endothelium causes swelling of the cornea. The essayist has confirmed this inasmuch as the cornea was swollen in the area of the endothelial curettage as compared with the untreated area. We have been able to demonstrate in the rabbit that the swelling of a corneal graft may be due to an endothelial defect. Before the implantation of the graft the endothelium was scraped off from one-half of the donor tissue. A microscopic section of the graft, four days after the operation, clearly showed the edematous swelling of the graft in the area where the endothelium was absent in contrast to the untreated area (*The Endothelium of the Cornea*. Tr. Am. Ophth. Soc. 1953, fig. 47).

Another important observation described by the essayist is the fact that the fibrin of the secondary aqueous while not adherent to the intact endothelium sticks to the denuded posterior surface of the cornea and to the surface of the iris and lens. The increased fibroblastic activity in the edematous cornea, after the initial phase of traumatic disturbance has subsided, tends to invade the fibrin plug and ultimately to cause permanent adhesions with the iris by connective tissue formation. The observation of increased fibroblastic activity in the edematous cornea is in accordance with what can be observed in humans during the late stage of Fuchs's corneal dystrophy where the cornea after longstanding edema becomes sclerosed (in the same place, fig. 31).

Of considerable interest to me was the essayist's observation that the fibroblasts on the posterior surface of the cornea tended to a certain polarity and became more parallel to the surface. This ties in with our own observation in monkey eyes (in the same place, figures 44, 45, 46). Fuchs as far back as 1917 (*Graefe's Arch. f. Ophth.*, 92:145-236) expressed doubt as to whether a regular endothelial cover could ever be regenerated after the endothelial cells have been destroyed. It seemed to us that the coverage of the wound gap might be achieved by proliferation of fibroblasts acquiring somewhat endothelial-like appearance without completely assuming the original pattern. On the other hand, it had been pointed out by Wagenmann (cited by von Hippel: F. Henke and O. Lubarsch, Berlin, Julius Springer, 1928) that the endothelium might produce connective tissue. Since the endothelium is probably of mesodermal origin, such substitutions would seem plausible.

As to the possible clinical implications which the author attributes to his findings there is one im-

portant reservation to be made. To what extent can the results of experiments made on rabbits be applied to human pathologic alteration. It is well known that the secondary aqueous of the rabbit contains enormously more fibrin than human aqueous. This factor is a source of constant annoyance in corneal grafting operations with rabbits. As shown by Magitot, the secondary aqueous of the rabbit contains 1500 mg. percent protein in contrast to only 180 mg. percent of the human secondary aqueous. One can hardly expect the fibrin in the human secondary aqueous to exert such a powerful adhesive force as was demonstrated by the author in the rabbit.

We tried to duplicate some of Dr. McDonald's experiments in cats whose aqueous still contains more protein than human aqueous but considerably less than rabbit. Of the five cat eyes on which an endothelial scraping was made on one side of the corneal incision only one eye had a flat chamber for three days after which it reformed. In the other four cases, the anterior chamber reformed promptly. Some fibrin was noted in the anterior chamber in all cases for a few days but in no instance did a fibroblastic organization of the clot occur and in no case did anterior synechias of the iris develop. It would appear that caution should be exercised in drawing hard and fast conclusions with regard to man from results obtained in rabbits. Nevertheless, I wholeheartedly agree with the essayist when he postulates meticulous avoidance of trauma to the corneal endothelium during intraocular surgery. Personally, I believe that miosis during the first few days after a

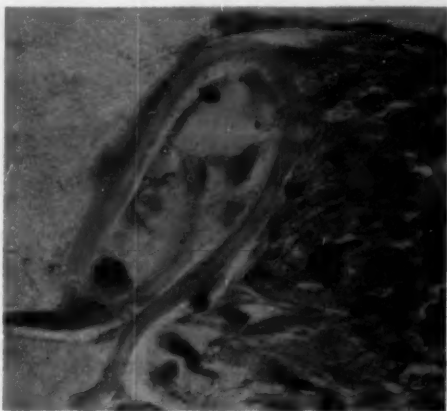


Fig. 1 (Stocker). To the right fibroblastic plug between the wound edges of a corneal section in the monkey. To the left curled up Descemet's membrane. Flattened cells, originating from the fibroblastic plug, cover Descemet's membrane in an endothelial like fashion. (Specimen courtesy of Drs. Dunnington and Regan.)

cataract extraction is important to keep the iris from sticking to the wound edges, and therefore I am opposed to indiscriminate use of atropine.

I want to congratulate Dr. McDonald on his fine work. I hope that he will be able to extend his studies to the question of the regeneration of the endothelium in general. Also, it would be most desirable if he could repeat some of the experiments in other animals, such as monkeys, thus operating under conditions resembling more closely those prevailing in humans.

DR. LUDWIG VON SALLMANN (Bethesda, Maryland): You did not distinguish, Dr. McDonald, between injury to the endothelium and injury to Descemet's membrane in addition to the wound in the endothelium. I don't know whether you have any proof that you injured the endothelium alone.

Experiments which we are carrying out in NINDB showed difference in the traumatic effect when the

wound involved only the endothelium or extended through Descemet's membrane into the stroma.

DR. FRANK W. POLACK (New York, New York): I want to ask Dr. McDonald whether he has made any attempt to use heparin or fibrolysin or dehydration of the cornea in the edematous side?

DR. JAMES E. McDONALD (closing): I wish to thank Dr. Stocker for his comments.

In regard to the application to human cataract surgery, we were quite cautious in the way we read this. We said, "Investigators might consider the role of adhesive forces between the iris and cornea as has been demonstrated by fibrin in these rabbit experiments." I have complete confidence that if these experiments were done on humans we would not get nearly the extent of fibrin adherences causing flat chambers. However, there is some considerable evidence that fibrin has a significant role in the closure of human corneal wounds.

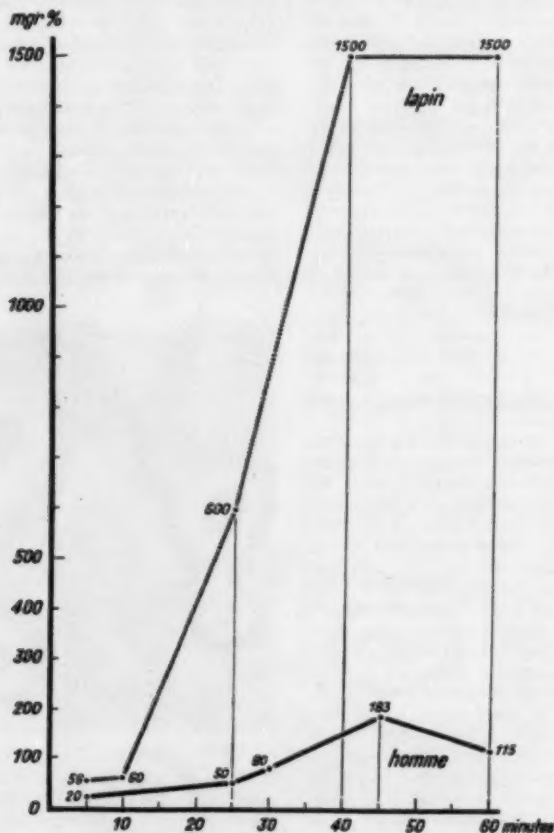


Fig. 2 (Stocker). The upper curve shows the tremendous increase of protein in the second aqueous of the rabbit, the lower curve the comparatively small increase in the human. (From Amsler et al. after Magitot.)

We have done experiments in reference to the fibrin phase of wound healing in humans. In four human cases that were about to be enucleated, central corneal incisions were made, and we found that the fibrin bridge was essential. The closure of this fibrin bridge was concomitant with the formation of the anterior chamber. This was in conjunction with a paper in which we used rabbits, cats, dogs and monkeys, both heparinized and non-heparinized, to establish this. (Rep. 17) A small chalazion curette was the instrument used to denude the endothelium.

In regard to Dr. von Sallmann's comments we were not able to distinguish by slit lamp between areas of endothelial damage and that of Descemet's damage. For example, on the control side of the incision there was no endothelial damage. But on the other side there were areas of normal endothelium (where the curette had missed), and areas where the endothelium alone was removed, and other areas where Descemet's membrane also was detached. So, perhaps this might better have been entitled, "The Effect of Curettage of the Back of the Cornea," without being so specific as to the endothelium alone.

THE DISTRIBUTION OF 5,5-DIMETHYL-2,4-OXAZOLIDINEDIONE (DMO) IN OCULAR AND CEREBROSPINAL FLUIDS

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Recent studies by Waddell and Butler¹ demonstrated that the intracellular pH of dog muscle could be determined by the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO). DMO is the metabolite of trimethadione (3,5,5-trimethyl-2,4-oxazolidinedione); it is an acid with a pK of 6.13, a value almost identical to that of carbonic acid. In accordance with the pH partition hypothesis put forth for gastric secretion by Shore et al.,² the ionized DMO would be distributed between compartments of fluids such that it would be in excess in the more basic fluid in similar fashion as bicarbonate. Since the intraocular fluids of rabbits have a higher pH and bicarbonate level than plasma, it was of considerable interest to determine whether DMO would be found in excess in these fluids. Similarly, spinal fluid which is acid relative to plasma,³ would show a deficit of DMO relative to plasma. The present report presents preliminary data on the study of the distribution of

oxazolidinediones in ocular and cerebrospinal fluids of rabbits.

METHODS

Male albino rabbits (Haskins Rabbitry) weighing 2.0 to 2.5 kg were used. Considerable care was taken to handle animals quietly and gently to avoid as much as possible excitement and acid-base shift. The experiments were done in the animal room so that the animals were in their cages in a familiar environment throughout the experiments. Paracenteses of posterior and anterior chamber were done with a 27 gauge needle with the eye proptosed under topical tetracaine anesthesia. Vitreous was obtained immediately after paracentesis of the anterior chamber by use of a plastic 2 ml syringe with a 20 gauge needle. Cerebrospinal fluid samples were obtained from the cisterna magna using a 1 ml plastic syringe and 22 gauge needle with a very short bevel immediately following death by intracardiac air embolus. Obtaining this fluid under ether anesthesia proved unsatisfactory because of the struggling of the animal during induction of anesthesia. The use of barbiturates was avoided as they interfere with the method. Heparinized plasma samples were obtained by cardiac puncture.

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TABLE I

THE DISTRIBUTION OF "DMO" IN OCULAR AND CEREBROSPINAL FLUIDS COMPARED TO PLASMA WATER FOLLOWING THE INTRAPERITONEAL ADMINISTRATION OF TRIMETHADIONE (400 MG./KG.)

Rabbit No.	Plasma mg. %	Pc Pl	Ac Pl	Vitr. Pl	Plasma mg. %	Pc Pl	Ac Pl	Vitr. Pl	CSF Pl
6 Hours					12 Hours				
1	8.7	1.07	0.81	0.63	21.5	1.08	0.80	0.88	0.73
2	14.4	1.03	0.83	0.71	35.0	1.21	1.01	1.00	—
3	12.4	0.95	0.71	0.81	33.2	0.91	0.71	0.72	—
4	11.6	0.87	0.73	0.52	24.6	1.07	0.87	0.73	0.68
	11.8	0.98	0.77	0.67	28.6	1.07	0.85	0.83	0.71
15 Hours					21 Hours				
2					52.4	—	—	—	0.87
3					47.4	—	—	—	0.93
11	36.8	1.17	0.89	0.82	54.8	1.25	1.02	1.17	0.80
12*	62.5	1.32 1.31	0.96 1.00	0.87 0.91	—	—	—	—	(0.77)
13	53.8	1.38	1.04	0.87	69.2	1.32	1.13	0.84	0.74
14	59.7	1.28	0.97	0.91	62.8	1.24	1.02	0.95	0.76
	53.0	1.29	0.97	0.88	57.3	1.27	1.06	0.99	0.82
27 Hours					33 Hours				
6	51.4	1.55	1.02	1.00	50.4	1.17	0.99	0.89	0.69
7	50.8	1.40	1.23	0.95	66.0	1.25	1.01	0.88	0.77
8	64.4	1.43	1.33	1.08	63.6	1.76	1.49	1.26	0.86
	55.5	1.46	1.19	1.01	60.0	1.39	1.16	1.01	0.77

* Animal died following heart tap and after parentesis of O.D.; O.S. was tapped immediately as well as CSF.

DMO* and trimethadione* were neutralized at a concentration of 150 mg/ml and 100 mg/ml, respectively. It was necessary to warm the solution of trimethadione to maintain this solubility. Paramethadione* is a liquid with a specific gravity of approximately 1.0. Trimethadione or paramethadione were given intraperitoneally at a dose of 400 mg/kg and DMO was similarly administered at a dose of 150 mg/kg alone or with 300 mg/kg of trimethadione. When not neutralized, the injection of DMO or trimethadione was very painful.

The analytical method was essentially that

of Butler⁴ proportionately decreased to permit analyses of 0.025 ml. All samples were taken with a calibrated Lang-Levy pipette with appropriate rinsing or washing and drying between samples. Final aliquots of 0.1 ml were read in microcells (0.05 ml) in the Beckman DU. As noted by Waddell and Butler,¹ DMO is not bound to albumin or muscle protein. The DMO equivalents in plasma of untreated rabbits is similar to that of dogs, 0 to 0.4 mg percent; that of aqueous is somewhat less, 0 to 0.2 mg percent, and have been ignored in the present calculations. An aliquot of 0.5 percent tetracaine equal to that of a sample (0.025 ml) was found to reduce the values by 2 mg percent. Consequently any contamination that might conceivably occur during parentesis would not interfere even with very low DMO values.

* Part of the DMO was obtained from K and K Laboratories. DMO, trimethadione and paramethadione were obtained through the courtesy of Dr. Schock of Abbott Laboratories. A sample of purified DMO for standards was kindly supplied by Dr. Waddell.

TABLE II

THE DISTRIBUTION OF "PMO" IN OCULAR AND CEREBROSPINAL FLUIDS COMPARED TO PLASMA FOLLOWING THE INTRAPERITONEAL ADMINISTRATION OF PARAMETHADIONE (400 MG./KG.)

Rabbit No.	Plasma mg. %*	Pc Pl	Ac Pl	Vitr. Pl	Plasma mg. %	Pc Pl	Ac Pl	Vitr. Pl	CSF Pl
15 Hours					21 Hours				
15	75.1	1.30	1.05	0.84	76.5	1.34	1.07	0.96	0.79
16	66.5	1.21	0.93	0.93	51.8	1.02	0.90	0.88	0.60
17	69.2	1.21	1.10	0.96	74.3	1.13	0.99	0.96	0.77
18	69.1	1.15	0.93	0.99	60.0	—	0.95	0.91	0.68
	70.0	1.22	1.00	0.93	65.7	1.16	0.97	0.93	0.71
29 Hours					35 Hours				
21	59.4	1.14	0.92	0.93	48.8†	1.22	1.00	0.96	—
22	57.8	1.21	0.83	0.87	57.0	1.21	1.05	0.99	0.72
23	68.5	1.26	1.11	1.12	68.0	1.26	1.24	1.08	0.86
24	44.5	1.04	1.04	0.94	39.7	1.05	1.09	0.99	0.60
	57.6	1.16	0.98	0.97	53.4	1.19	1.10	1.01	0.73

* Measured as "DMO."

† Value was 50.3 at 32 hrs.

RESULTS

Data on blood levels and distribution of DMO in ocular and cerebrospinal fluids following the administration of trimethadione are summarized in Table I and Figure 3. The accumulation of DMO in the plasma reached a plateau in approximately 15 to 21 hours. The balance between the demethylation of trimethadione to form DMO and the excretion of DMO resulted in a reasonably stable blood level averaging 56 to 60 mg percent for at least 12 hours. The concentration of DMO in posterior aqueous was in excess of that of plasma even during the rising blood level phase. The excess concentration approached a

steady state level which averaged 43 percent at 27 to 33 hours. Assuming a plasma pH of 7.40 this excess would correspond to a pH of 7.56 (fig. 2). DMO was not found in excess in the anterior aqueous in a majority of eyes until after 15 hours. The anterior aqueous concentration of DMO was higher than that of plasma by an average of 18 percent at 27 to 33 hours, corresponding to a pH of 7.48. The level of DMO in the vitreous rose considerably more slowly. It approached plasma level at 21 hours and was an average of one percent in excess at 27 and 33 hours. The level of DMO in cerebrospinal fluid was in deficit compared to plasma in all animals. This deficit aver-

"DMO"

5,5-Dimethyl-2,4-oxazolidinedione

Trimethadione (TMO)

3,5,5-Trimethyl-2,4-oxazolidinedione

Paramethadione (PMO)

3,5-Dimethyl-5-ethoxazolidine-2,4-dione

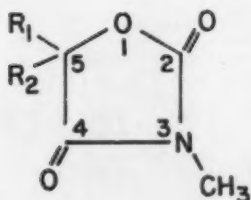


Fig. 1

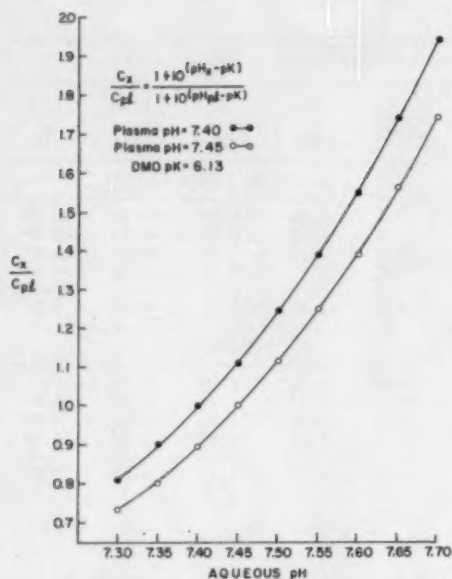


Fig. 2

aged 19 percent at 21 hours and 23 percent at 33 hours and would correspond to an average pH of 7.29, if the plasma pH were 7.40.

The blood level of the demethylated paramethadione (PMO) appeared to plateau earlier than that of the metabolite of trimethadione (table II, and fig. 4). Since purified PMO was not available, the concentrations were measured as DMO. This oxazolidinedione behaved in similar fashion as DMO with excesses of some 20 percent in the posterior chamber, 10 percent in the anterior chamber and a deficit in cerebrospinal fluid. Since the pK value of PMO is not available at this time, pH values corresponding to these distribution ratios cannot be calculated.

Following the intraperitoneal injection of DMO the blood level (table III, fig. 5) showed an early peak with a small decrease from one to three hours and remained reasonably stable from the third to seventh hour. The excess concentration in the posterior chamber compared to plasma averaged 23 percent and in the anterior aqueous averaged 9 percent at 7.5 hours. The vitreous level was 99 percent of plasma at this time. The amount of DMO in cerebrospinal fluid was in deficit by 21 percent. The pH values corresponding to the level of DMO in ocu-

TABLE III

THE DISTRIBUTION OF "DMO" IN OCULAR AND CEREBROSPINAL FLUIDS COMPARED TO PLASMA FOLLOWING THE INTRAPERITONEAL ADMINISTRATION OF "DMO" OR "DMO" PLUS TRIMETHADIONE*

Rabbit No.	Plasma mg. %	Pc Pl	Ac Pl	Vitr. Pl	Plasma mg. %	Pc Pl	Ac Pl	Vitr. Pl	CSF Pl
DMO— 3 Hours					7½ Hours				
35	31.7	1.19	1.06	1.04	32.9	1.22	1.04	0.90	0.75
36	32.7	1.04	1.08	0.90	32.7	1.20	1.04	1.01	0.67
37	32.4	—	0.93	0.73	30.7	1.18	1.09	0.95	0.83
38	33.0	1.27	1.18	0.93	32.8	1.30	1.20	1.08	0.90
	32.5	1.17	1.06	0.90	32.3	1.23	1.09	0.99	0.79
DMO + TMO— 5½ Hours					12 Hours				
31	47.1	1.26	1.10	0.90	72.2	1.20	1.14	0.89	—
32	47.5	1.09	1.06	0.89	—	—	—	—	(0.80)
	48.6†	1.23	0.96	0.86					
33	46.4	1.22	0.99	0.82	63.3	1.22	1.20	0.87	0.76
34	44.3	1.22	1.06	0.91	70.8	1.14	1.12	0.95	0.73
	46.8	1.20	1.03	0.88	68.8	1.19	1.15	0.90	0.75

* 150 mg./kg. "DMO" alone or with 300 mg./kg. TMO.

† 6½ Hours; This eye had a deep anterior chamber. However, since the animals were not examined by slit lamp, the data are included.

CONCENTRATION OF "DMO" AFTER TRIMETHADIONE I.P.

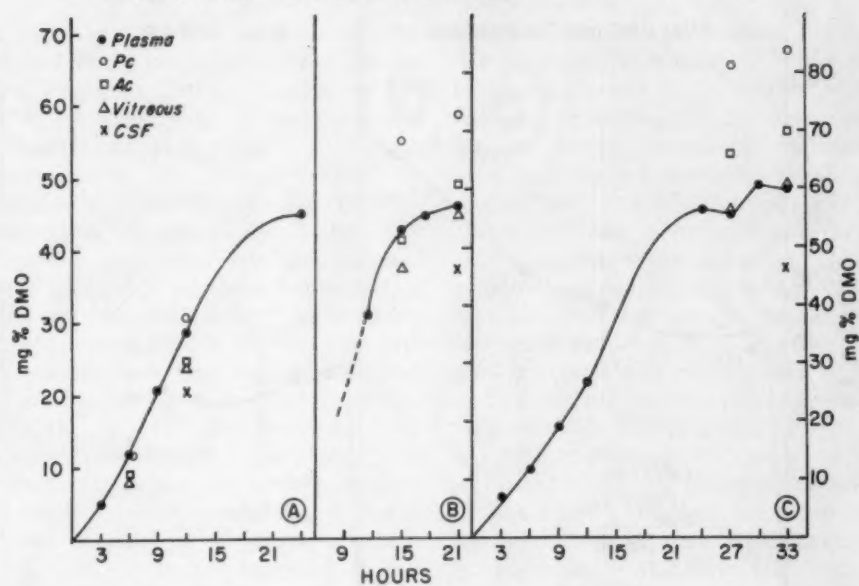


Fig. 3

CONCENTRATION OF "PMO" AFTER PARAMETHADIONE I.P.

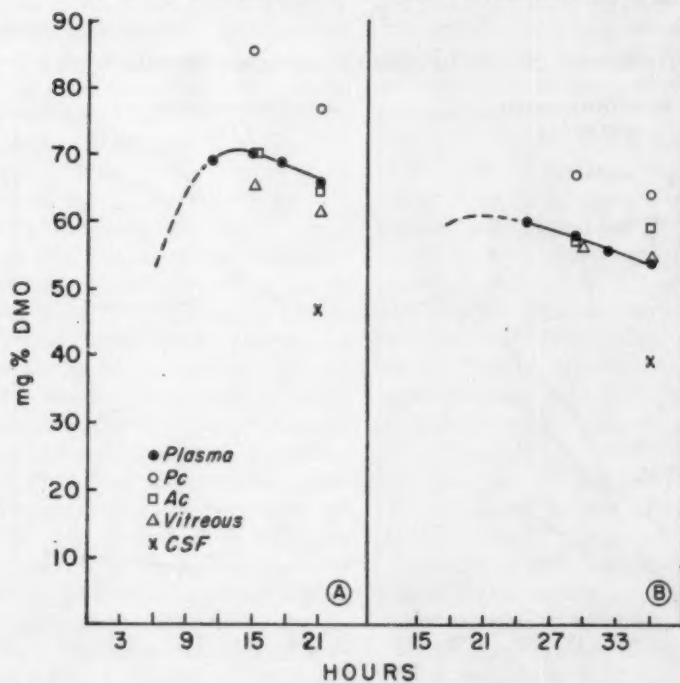


Fig. 4

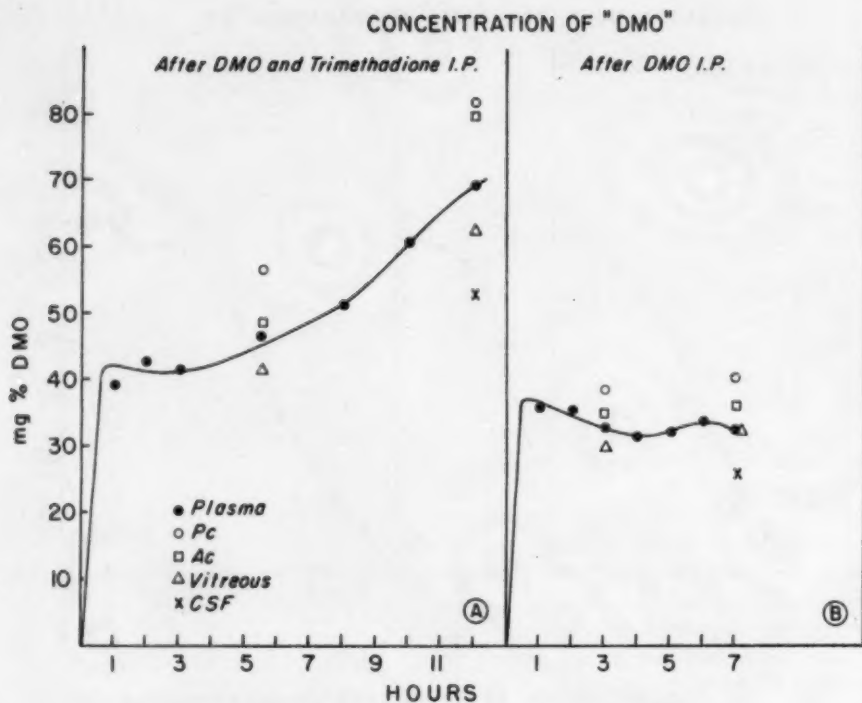


Fig. 5

DISTRIBUTION OF DMO IN OCULAR AND CEREBROSPINAL FLUIDS

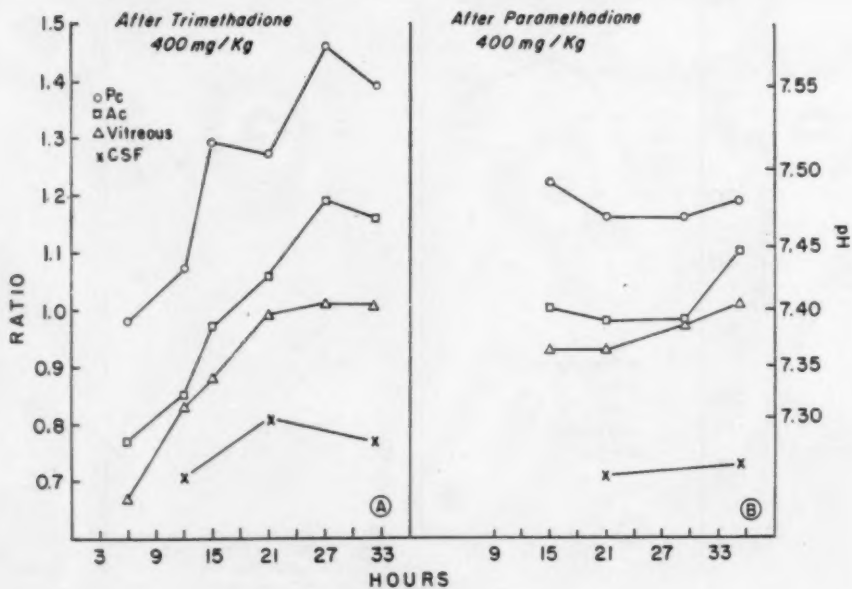


Fig. 6

lar fluids at 7.5 hours would be approximately 7.50 for posterior aqueous, 7.44 for anterior aqueous, 7.39 for vitreous and 7.29 for cerebrospinal fluid on the assumption that the plasma pH was 7.40.

Although in the present studies, the plasma level of DMO following DMO injection was reasonably steady for four hours, previous experience had indicated that a rapid fall could be anticipated at longer experimental periods. Unfortunately the supplemental level of trimethadione used was greater than that required to offset the anticipated DMO loss by excretion; (table III, fig. 1); consequently, the blood level began to rise after four hours. The excesses in posterior and anterior aqueous and deficit in cerebrospinal fluid are similar to those obtained in the previous experiments.

DISCUSSION

Since the distribution of DMO follows pH, it is obvious that the maintenance of stable plasma levels in the rabbit would be affected not only by shifts in acid-base balance but also by excretory loss into the normally alkaline urine. In the present studies it was found that the careful handling of animals and the neutralization of the drugs helped to avoid part of this problem. The combined use of DMO and trimethadione seem promising in minimizing the effect of urinary loss in the intact animal.

Although the maintenance of a steady blood level for long periods presents a problem and needs further study, the use of DMO appears to be an exciting tool for ophthalmic research. The excesses of DMO in posterior and anterior aqueous observed in these studies provide data for calculated pH values which are in reasonable agreement with those obtained by colorimetric⁶,⁸ or glass electrode⁷ methods. The calculated pH value for the posterior aqueous was 7.56; that of the anterior aqueous slightly less, 7.48. Although the lower value for anterior aqueous is somewhat at variance with the data of Kinsey,⁸ who found no signifi-

cant difference between anterior and posterior aqueous pH, it does reflect the lower bicarbonate content of the anterior aqueous and may be the true pH for these rabbits. Studies directly comparing colorimetric, glass electrode and the DMO methods are contemplated. The cerebrospinal fluid was always in deficit in comparison to plasma values to a degree that appeared to be primarily related to the individual animal. The approximate deficit of 20 percent would correspond to a pH of 7.29 which is in good agreement with values quoted by Davson.⁹ These pH values were calculated on the assumption that the average plasma pH was 7.40. In most animals spinal taps were performed within one to two minutes after air was injected into the heart; however, it is possible that some shift of DMO occurred in this interval. Although DMO studies present some problems because of steady state difficulties, the beauty of the method is that it measures pH at the temperature of the fluids *in vivo*, is not affected by temperature changes or carbon dioxide loss after removal from the body, nor is it affected by protein error, as are colorimetric or glass electrode methods.

The observations that the excess of DMO in the posterior aqueous occurred during the rising blood level, and that of the anterior aqueous was attained much more slowly and failed to reach the levels of the posterior aqueous (although the pH of the two fluids are reported to be similar), tempt one to speculate that the entrance of DMO into the eye may be in part a consequence of a secretory process. In this regard, the study of animals with different secretory rates either normally or produced by secretion inhibitors, and the study of higher and considerably lower plasma levels should prove most interesting. Tonographic data obtained after single or daily injections of these compounds did not demonstrate any consistent effect on aqueous flow at the present dose level.

Preliminary studies have indicated that DMO penetrates the lens in sufficient

quantity to permit its detection. However, the high protein content of this organ presents some analytical problems. It is hoped that further study of the analytical procedure will result in the development of a sufficiently micro method to permit one to study the fascinating problems of DMO concentration changes in the lens with cataract development and in the ciliary body after the administration of secretion inhibitors.

Although the present studies are preliminary, it is felt that these oxazolidinedione compounds are of sufficient potential and interest to ophthalmic research to warrant the publication of the data at this time.

SUMMARY

The blood levels and distribution in posterior, anterior, vitreous and cerebrospinal

fluids of oxazolidinedione compounds were studied. The compounds used were 5,5-dimethyl-2,4-oxazolidinedione (DMO) 3,5,5-trimethyl-2,4-oxazolidinedione and 3,5-dimethyl-5-ethoxazolidinedione. The demethylated metabolites of these drugs were observed to be in excess in the posterior and anterior aqueous and in deficit in the cerebrospinal fluid. The pH values calculated from the ratio of the concentration of DMO in posterior aqueous and cerebrospinal fluid compared to plasma are in good agreement with reported values obtained by colorimetric and glass electrode methods. The data obtained in the present study suggest that the entrance of DMO into ocular fluids may be in part a consequence of a secretory process.

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EXPERIMENTAL INTRAOCULAR MALIGNANT MELANOMA IN THE SYRIAN GOLDEN HAMSTER

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There are some important clinical problems concerning human malignant melanoma of the eye which may be studied by a reproducible animal model of the disease. Among these are the difference in malignancy of melanomas of the iris and choroid, the value of surgical excision or chemotherapy of a tumor bearing eye, the effect of photocoagulation of tumors, antigen-antibody relations, enzyme or isotope uptake studies and others. The purpose of this paper is to describe the course of two different types of spontaneously occurring cutaneous malignant melanomas of the Syrian Golden hamster when transplanted into the anterior chamber of the hamster eye and to describe preliminary results of therapy of malignant melanoma by enucleation of a tumor-bearing eye.

As early as 1882 tissues were transplanted to animals,¹ but the value of the anterior chamber of the animal eye as a favorable site where growth could be visualized was emphasized when H. S. N. Greene succeeded in obtaining growth of various mammalian and human tumors in the anterior chamber of laboratory animals.² Since then much work has been done with the anterior chamber homologous and heterologous transplants.

Most tumor transplants into anterior chambers have been done with solid pieces of tumor. Since it is difficult to know how much of a tumor block is connective tissue, necrotic tumor, or viable tumor, this makes for large variation between blocks and since the eye is so small, it seemed that injection of a measured volume of a suspen-

sion of tumor cells might be a more reproducible and accurate technique. With injection of a suspension a 30-gauge needle can be used instead of the usual trocar and a much smaller inoculum can be obtained by dilution.

Recently transplantable spontaneous malignant melanomas have been described in Syrian Golden hamsters.^{3,4} It has been suggested that these tumors developed from junctional nevi.⁴ These tumors have been serially transplanted and metastasize from subcutaneous transplantation sites to cause death of the animal. We were fortunate in being able to obtain these tumors via subcutaneous transplants and have compared a tumor, furnished by Dr. H. S. N. Greene, Yale University, New Haven, Connecticut, with one of three melanomas provided by Dr. Joseph G. Fortner, Memorial Center for Cancer and Allied Diseases, New York City. These will be termed the Greene and Fortner tumors, respectively.

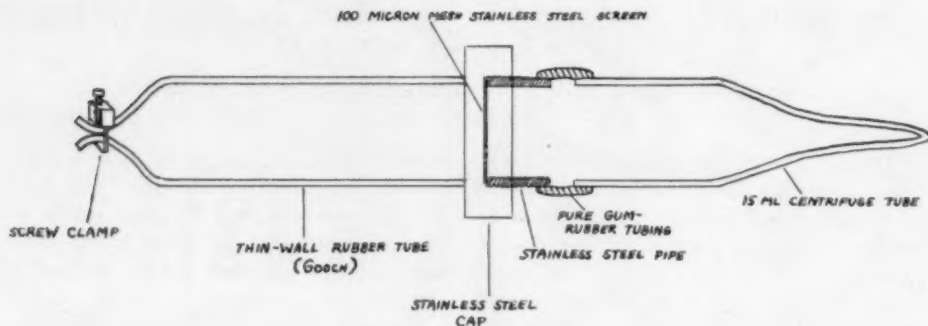
MATERIALS AND METHODS

Approximately 200 Syrian Golden hamsters,[†] weighing from 65 to 95 grams, have been utilized to date. The animals are housed in wire cages and maintained on fresh vegetables, Purina lab rat chow and water ad libitum.

The tumor material was transferred under sterile conditions within four hours after removal from the host. The subcutaneous tumor was weighed in a tared dish, minced, diluted with a balanced salt solution, broken up in a Snell cytosieve⁵ and filtered through a stainless steel wire screen of 100 microns mesh size (fig. 1). This resulted in a suspension with multiple single cells and small clumps of cells (fig. 2), which could

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† Obtained from Roberta's Hamstery, 8505 S. E. Yamhill St., Portland, Oregon.



CROSS-SECTION OF MODIFIED SNELL CYTOSIEVE

Fig. 1 (Burns and Fraunfelder). A piece of tumor and diluting fluid is placed in the Gooch tubing, minced manually, forced through the screen to the centrifuge tube and aspirated through the gum rubber sleeve into a syringe for injection.

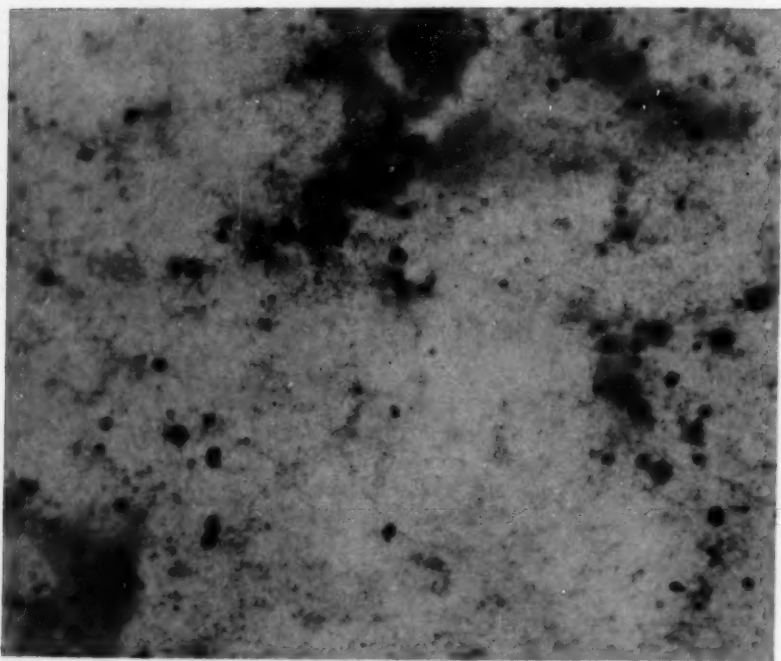


Fig. 2 (Burns and Fraunfelder). Tumor suspension prepared in Snell cytosieve from Greene tumor. Suspension is principally single cells. Hematoxylin-eosin of thin smear, magnified 300 X.

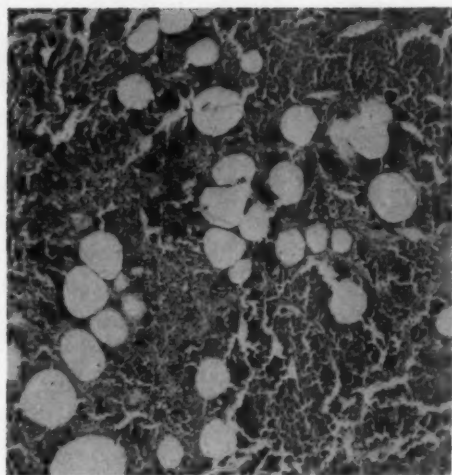


Fig. 3 (Burns and Fraunfelder). Greene tumor, subcutaneous implant. Viable cells surrounded by necrotic areas. Pigment tends to clump at the junction of the active tumor with necrotic tissue. Hematoxylin-eosin magnified 150 \times .

not be counted accurately due to grouping of cells.

The animals were anesthetized with 0.01 cc. per gram of weight of a mixture of commercial pentobarbital (nembutal-Abbott) diluted with 8.5 parts 10 percent ethanol, injected intraperitoneally. The cornea was anesthetized with topical 0.5 percent tetracaine. Under 45 \times magnification by a dissecting microscope, a slanting incision was



Fig. 5 (Burns and Fraunfelder). Normal hamster iris and chamber angle. Note large episcleral vessels. Magnified 150 \times .

made with a dissection knife just inside the limbus at the superior margin of the cornea. An air bubble was injected to fill the anterior chamber and the tumor cell suspension injected with a Hamilton 0010 $\frac{1}{2}$ microsyringe, using a blunt 30-gauge needle. Volume of injection ranged from 0.004 to 0.0023 ml. Frequently some of the injected suspension flowed back through the corneal incision. This loss of injection was possibly minimized by air bubble injection and by a beveled incision, with careful attention to atraumatic withdrawal of the injection

* Obtained from the Hamilton Company, P.O. Box 307, Whittier, California.

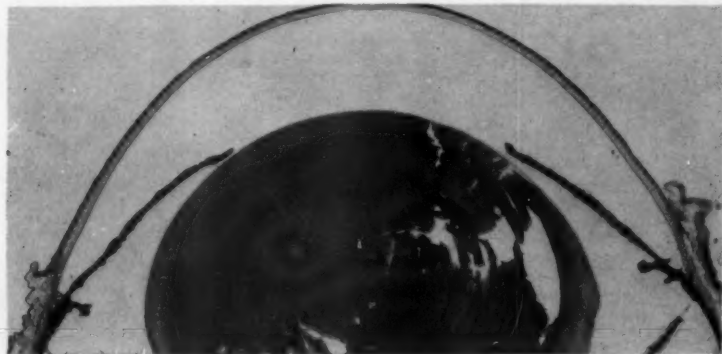


Fig. 4 (Burns and Fraunfelder). Normal hamster anterior segment. Iris is heavily pigmented with no cellular detail visible. Lens large in proportion to eye. Hematoxylin-eosin, magnified 35 \times .

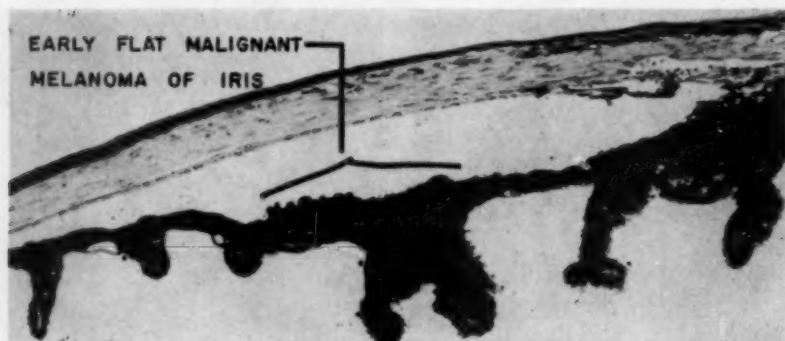


Fig. 6 (Burns and Fraunfelder). Tiny plaque of cells at site of implant of Greene tumor, ten days after injection of cell suspension. Hematoxylin-eosin, magnified 150 \times .



needle and avoidance of pressure on the animal's head and eye. The eyes were examined with the microscope at regular one to two week intervals under intraperitoneal pentobarbital-ethanol anesthesia. In the first animals, both eyes were injected to perfect technique but the last 120 animals were injected in one eye only.

Fig. 7 (Burns and Fraunfelder). Drawing of early nodule of Greene tumor of iris. Drawings employed because photography of brown tumor on brown iris unsatisfactory.

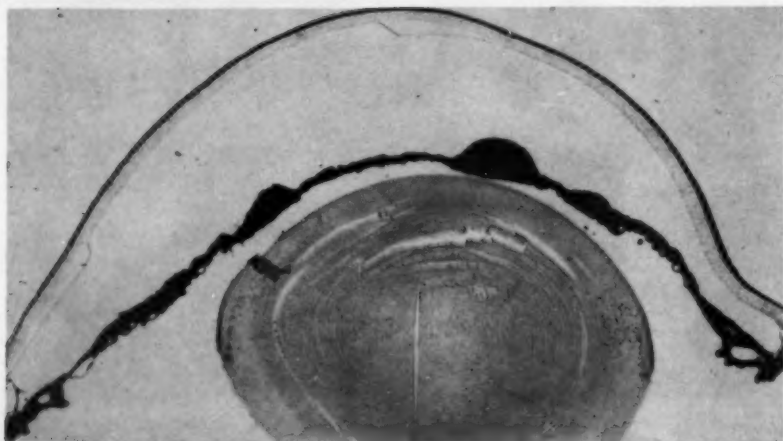


Fig. 8 (Burns and Fraunfelder). Three separate nodules of Green tumor of iris. Hematoxylin-eosin, magnified 45 \times .



Fig. 9 (Burns and Fraunfelder). Drawing of confluent spreading masses of Greene tumor which has not yet reached the periphery of the iris.

RESULTS

I. Greene tumor

The Greene tumor is grossly black. Microscopically, it is a highly anaplastic malignant melanoma of epithelioid cell type,

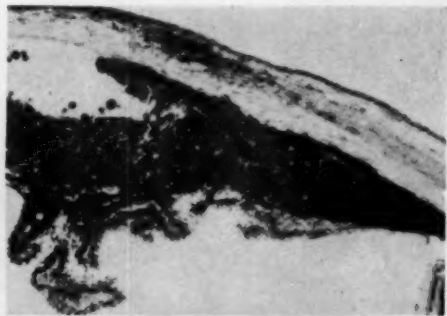


Fig. 11 (Burns and Fraunfelder). Extension of Greene tumor from iris into episcleral vessels (compare fig. 5) through trabecular area. Hematoxylin-eosin, magnified 150 \times .

growing in sheets and cords. Some nests of cells resemble nevi. Necrosis is frequent. It is moderately pigmented, more heavily near necrotic areas (fig. 3). There is a moderate reticulin content.

During the first week after injection the animal's eyes show no evidence of neoplasm. There may be varying degrees of iris adherence to the corneal wound, iris laceration or hemorrhage, or traumatic cataract, but this was less often seen as technique improved. As early as eight days, minute tu-

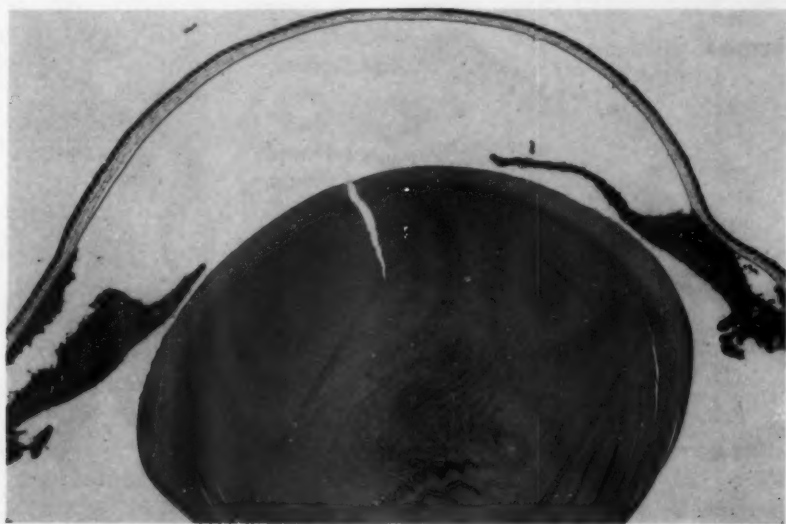


Fig. 10 (Burns and Fraunfelder). Greene tumor diffusely spread over iris, involving the chamber angle. Hematoxylin-eosin, magnified 45 \times .

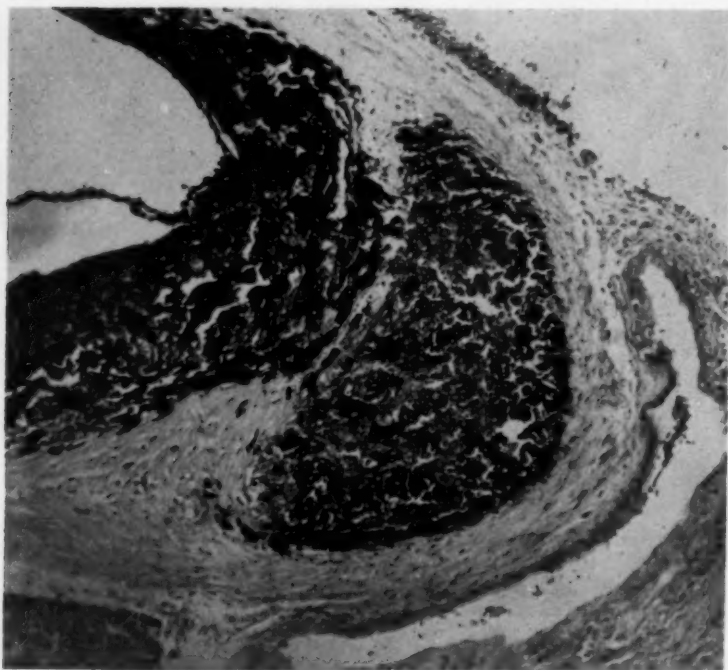


Fig. 12 (Burns and Fraunfelder). Extrascleral extension of Greene tumor to form limbal nodule. Conjunctival sac at lower right. Hematoxylin-eosin, magnified 150 \times .

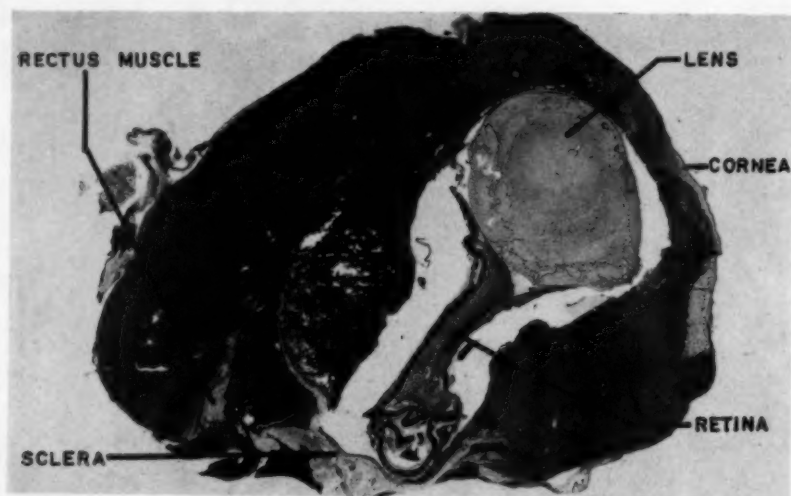
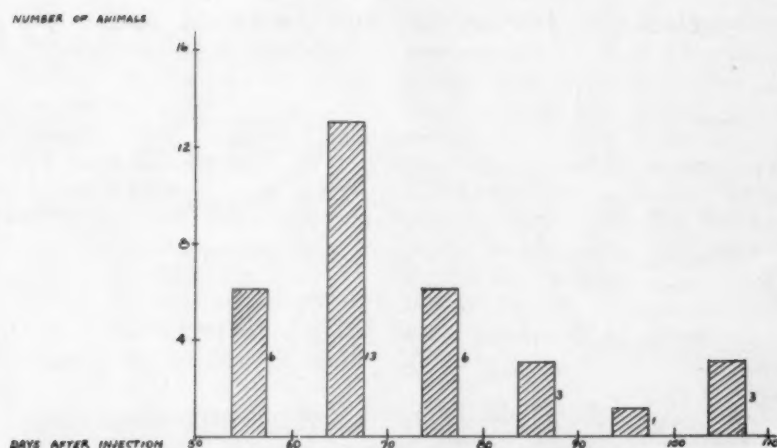


Fig. 13 (Burns and Fraunfelder). Replacement of eye and orbital contents by Greene malignant melanoma extending from anterior chamber injection. Hematoxylin-eosin, magnified 35 \times .



**LENGTH OF SURVIVAL OF HAMSTERS
INJECTED INTRAOCULARLY WITH GREENE TUMOR**

Fig. 14 (Burns and Fraumfelder). In hamsters in which tumor take was obtained, death occurred between 54 to 103 days. The mortality rate is maximal in the second month.

EFFECT OF DILUTION ON TUMOR TAKE

(AMOUNT INJECTED VARIED FROM 0.0004 TO 0.002 ML.)

DILUTION OF CELL SUSPENSION	TAKES	NUMBER OF ANIMALS NO TAKES	TOTAL
1:1	14	0	14
1:5	54	12*	66
1:10	11	2	13
1:50	1	1	2
1:500	4	2	6
1:5000	0	4	4
TOTAL	84	21	105

* ALL WERE 0.0004 ML. INJECTIONS

Fig. 15 (Burns and Fraumfelder). If injection is large enough, ID 50 of Greene tumor suspension is 1:500 dilution.

EFFECT OF SIZE OF INOCULUM ON TUMOR TAKE

(DILUTIONS FROM 1:1 TO 1:5000)

AMOUNT INJECTED (ml.)	TAKES	NUMBER OF ANIMALS NO TAKES	TOTAL
0.0023	5	0	5
0.0016	8	8*	16
0.0008 TO 0.001	42	1**	43
0.0004	29	12***	41
TOTAL	84	21	105

* INCLUDES THREE 1:5000 AND TWO 1:500

** INCLUDES ONE 1:5000

*** ALL WERE 1:5 DILUTION

Fig. 16 (Burns and Fraunfelder). Either too small a volume or too high a dilution of suspension may cause failure of tumor take.

mors may be seen with the dissecting microscope on the iris, as light brown flat patches occurring in isolated areas, which can be seen microscopically as plaques of cells (figs. 4, 5, 6). These grow to form nodules (figs. 7, 8), and spread over the iris,

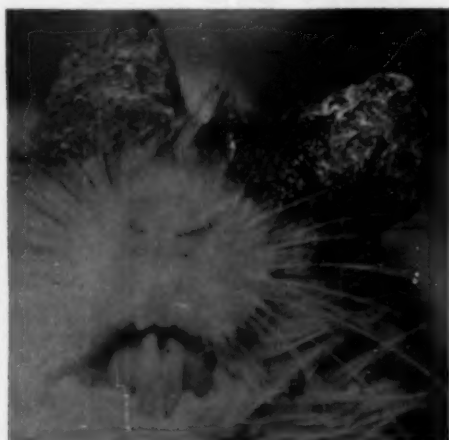


Fig. 17 (Burns and Fraunfelder). Hamster with eyes replaced by large tumors. Both eyes were injected with suspension of Greene malignant melanoma.

become confluent, destroy the iris architecture, fill the chamber angle and anterior chamber (figs. 9, 10). The cornea turns cloudy as glaucoma presumably ensues from angle blockage. From the chamber angle microscopic spread through the trabecular area occurs (fig. 11). Within 20 to 30 days nodules appear outside the eye, either at the limbus or corneal incision (fig. 12). The conjunctiva and eyelids are invaded by direct extension and the eye is replaced by a mass of tumor tissue six to 13 millimeters in size (fig. 13). Within 40 to 60 days palpable cervical nodes appear, and death occurs from 54 to 103 days (fig. 14).

Two-thirds died within 55 to 75 days, and 0.9 by 85 days. One reason for the spread of data is that successful injections of tumor suspension in this series vary in dilution from 1:1 to 1:500 (fig. 15) (no takes were obtained in four animals injected with 0.0008 to 0.001 ml. of 1:5000 dilution) and in volume from 0.0004 to 0.0023 ml. (fig. 16). In the first 105 eyes, takes were obtained in 84, or 80 percent and no sex dif-

ference was observed. Either too small a volume, or too great a dilution, of the injection increased the incidence of failure. Probably an injection of 0.001 ml. of a 1:5 dilution will give almost 100 percent takes with discrete tumor nodules.

In one animal a tumor take was not visualized until 45 days after injection but the tumor seemed to grow at the usual rate after becoming microscopically visible.

Autopsy shows the tumor spreads by both lymph and blood borne metastases, as well as by direct extension. The eyes are fungat-

ing black conical masses (fig. 17). The tumor grows by direct extension into the eyelids, orbit and cranial cavity (fig. 18). Posterior auricular, cervical and thoracic cage nodes are involved by lymphatic spread. Often the hilar nodes are so enlarged that they fill over one-fourth of the total thoracic cavity and cause the animal to breath by bending the spine, since the diaphragm and intercostal spaces appear to be frozen (fig. 19). Extensive thoracic wall and diaphragmatic metastases occur (Fig. 20). The lungs are always involved (fig.

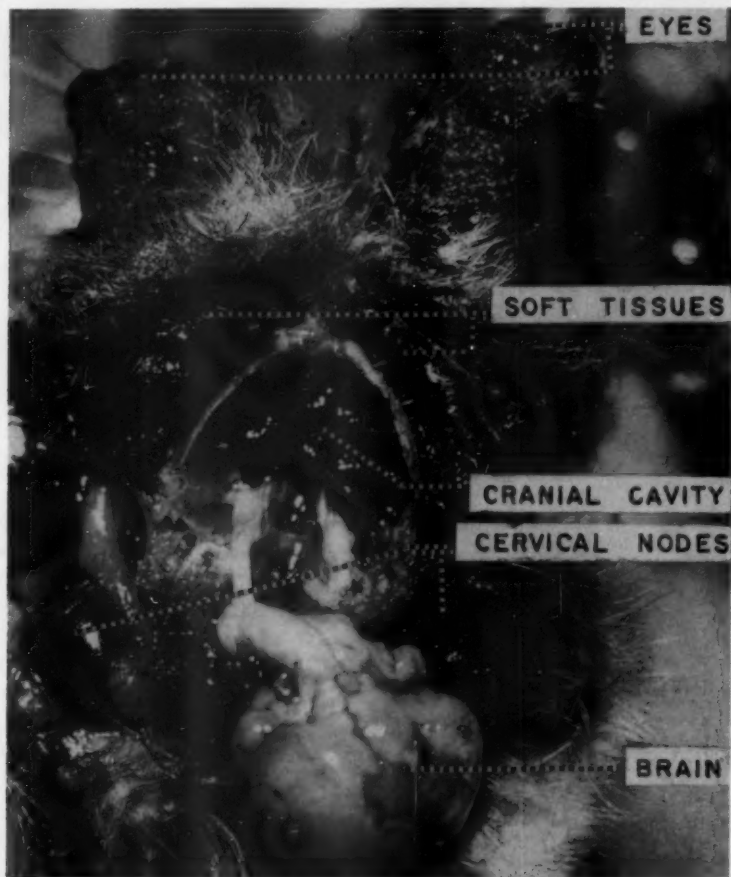


Fig. 18 (Burns and Fraunfelder). Dorsum of head with top of skull removed and brain reflected. Tumor involves the eyes, soft tissue around eyes and cranial cavity by direct extension.

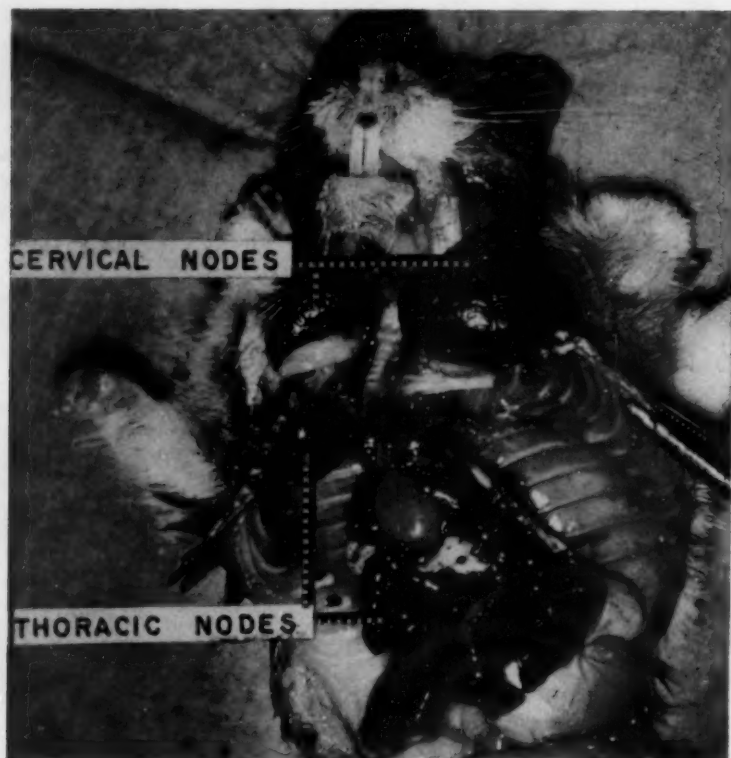


Fig. 19 (Burns and Fraunfelder). Extensive black metastases in cervical and posterior thoracic nodes, probably due to lymphatic spread.

21). Metastases below the diaphragm are rare but a few liver metastases have been found. Nearly all the animals are cachectic at death.

II. Fortner tumor

Grossly, the Fortner tumor is light gray-tan. It is also an epithelioid malignant melanoma, growing mostly in sheets of cells, with very little pigment except in the older lesions (fig. 22). Necrosis is prominent and reticulin content moderate.

Growth of tumor in the anterior segment of the eye probably appears less rapidly than with the Greene tumor but the earliest lesions are lighter colored and more visible. At eight to 12 days the tiniest light tan dots are visible on the iris (figs. 23, 24). However, these grow very slowly, eventually acquiring visible vessels and increased

pigmentation (fig. 25). The tumor nodules coalesce to almost fill the anterior chamber (fig. 26) but show little tendency to lateral spread over the iris to cover the pupil or angle (fig. 27). It is notable that the cornea remains clear with the anterior chamber 90 percent full of tumor, indicating that glaucoma has not been produced by chamber angle or pupillary blockage. Tumor takes have been obtained in 18 of 21 eyes, both male and female, injected to date and with improvement in technique probably 100 percent takes will occur. Visible tumor take was delayed to 75 to 80 days after injection in one small animal.

A lesser tendency to spread outside the eye occurs with the Fortner tumor than with the Greene tumor (fig. 28). Extrascleral extension of the tumor into the sub-

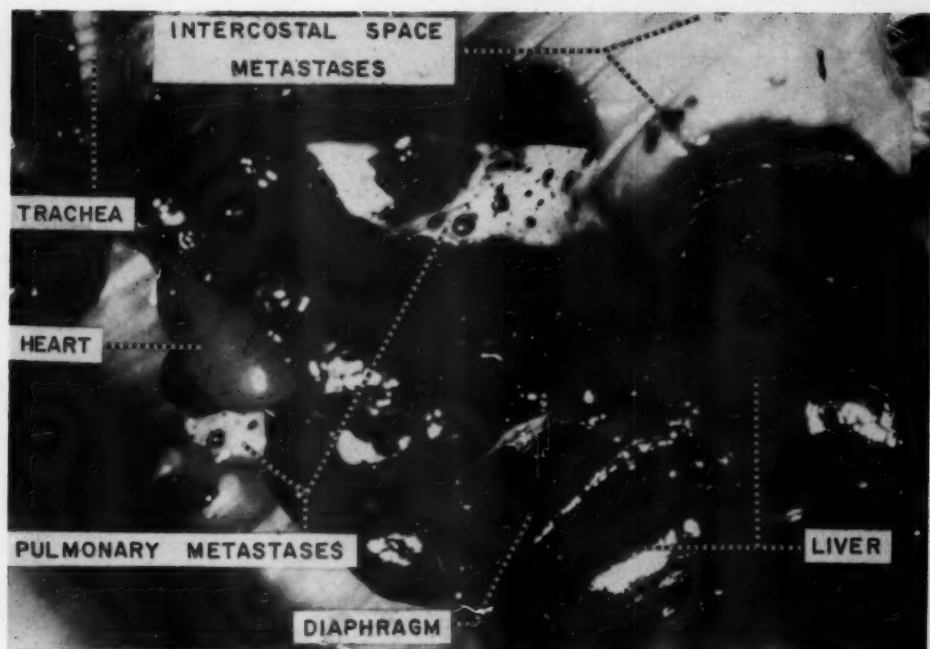


Fig. 20 (Burns and Fraunfelder). Numerous small black nodules of metastasis in lungs, probably by blood-borne spread. Metastases in posterior thoracic nodes much larger than pulmonary or intercostal space metastases.

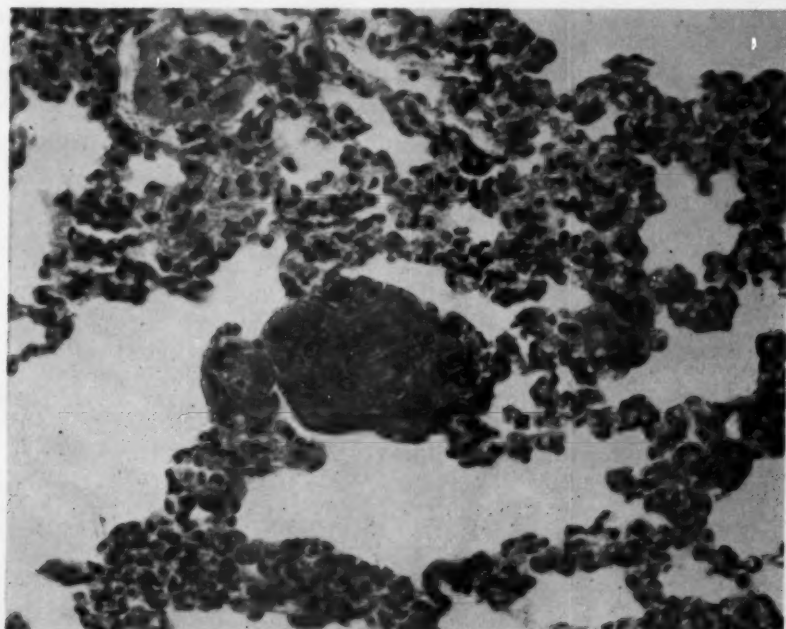


Fig. 21 (Burns and Fraunfelder). Microscopic pulmonary metastasis of Greene tumor, showing melanin in periphery. Hematoxylin-eosin, magnified 300 \times .

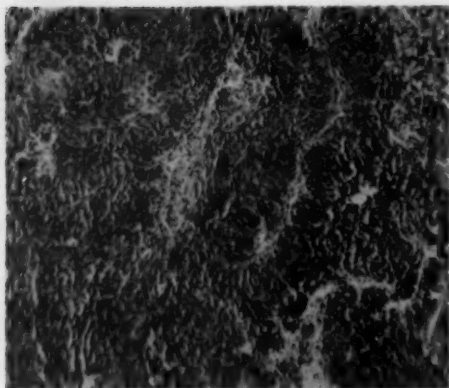


Fig. 22 (Burns and Fraunfelder). Fortner malignant melanoma, subcutaneous implant. Sheets and cords of epithelioid cells, with sparse melanin pigment. Hematoxylin-eosin, magnified 150 \times .

conjunctival tissue occurs at 56 to 70 days but no cervical metastases have been found. One animal died from metastases at 107 days, but nine others have remained healthy over 120 days, including three animals whose tumor bearing eye was enucleated. One healthy animal killed at 142 days had the eye replaced by tumor with extension from iris to choroid and orbit (fig. 29) and had multiple localized intrapulmonary and subpleural metastases.



Fig. 24 (Burns and Fraunfelder). Larger growth of Fortner tumor of iris. No tendency to lateral spread. Hematoxylin-eosin, magnified 150 \times .

III. Enucleation

The effect of enucleation of a tumor bearing eye on survival of human patients with malignant melanoma has been a disputed topic. It was decided to enucleate eyes after tumor growth had been visibly established and verify the presence of malignant melanoma microscopically. Preliminary results indicate that a high percentage of animals with Greene tumor survive after enucleation of a tumor bearing eye, while all control animals die in the expected time. This work is continuing and the value of the Meyer-Schwickerath technique of treatment of malignant melanoma with the Zeiss photocoagulator will be studied.

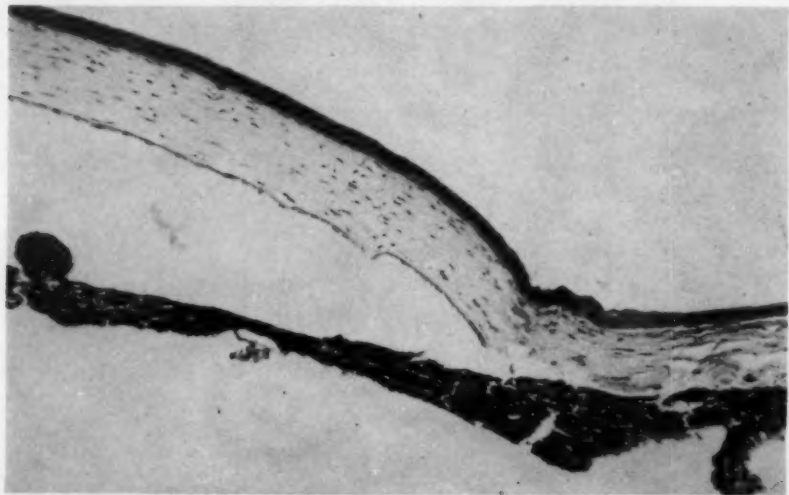


Fig. 23 (Burns and Fraunfelder). Early growth of Fortner tumor of iris. Nodules easily visible with dissecting microscope due to light color. Hematoxylin-eosin, magnified 150 \times .

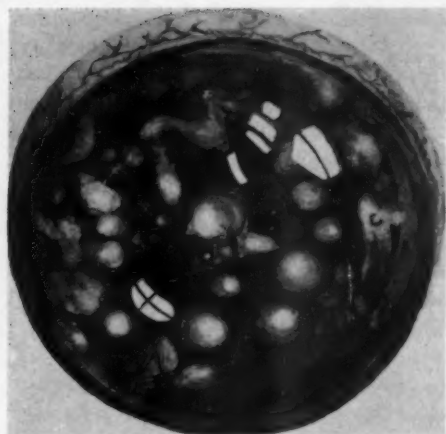


Fig. 25 (Burns and Fraunfelder). Discrete gray-white isolated nodules of Fortner tumor growing on iris.

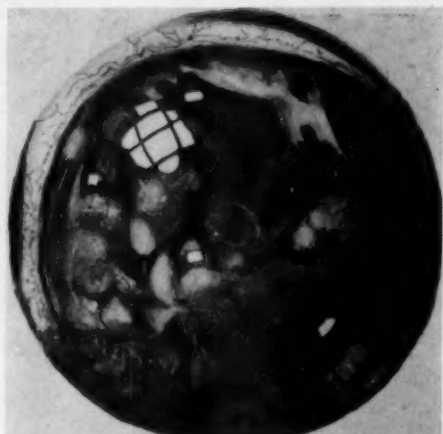


Fig. 26 (Burns and Fraunfelder). Coalescent large masses of Fortner tumor with increase in pigment and vascularity.

IV. Miscellaneous

Since there is difficulty keeping the total dose of injected tumor in the anterior chamber, attempts have been made to increase the viscosity of the tumor suspension by

adding methylcellulose or vitreous, by decompression of the vitreous cavity or posterior chamber, or injection through a sharp needle without a preliminary discission knife incision. No method has been found to be

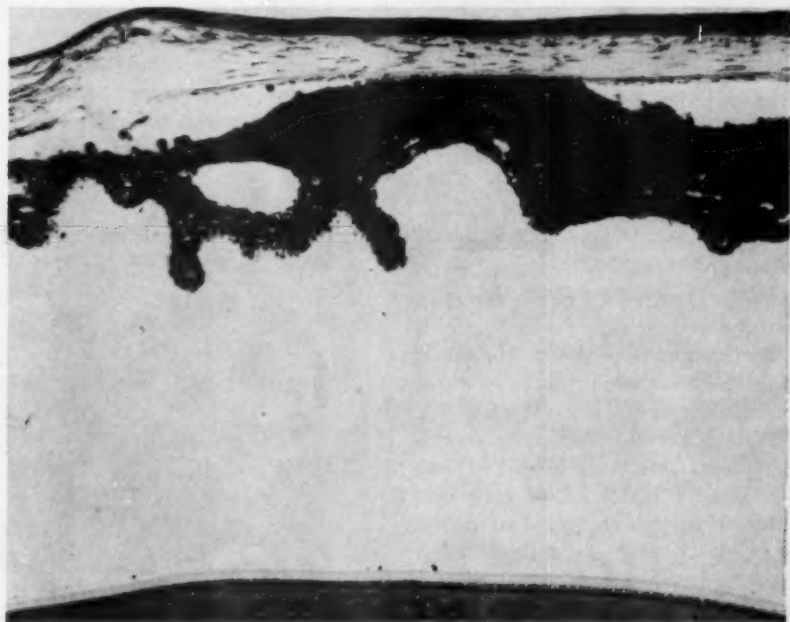


Fig. 27 (Burns and Fraunfelder). Fortner tumor of iris. This nodule touches cornea but has not spread into the chamber angle. Hematoxylin-eosin, magnified 150 \times .



Fig. 28 (Burns and Fraunfelder). Enucleated eye showing spread of Fortner tumor posteriorly from iris to choroid without escaping confines of eye. Hematoxylin-eosin, magnified 35 \times .

completely satisfactory but with experience more consistent results were obtained. With the method described in this paper, we have found that an injection of 0.001 ml. of a 1:5 dilution of Greene tumor suspension resulted in takes in almost 100 percent of animals.

No tumor take was found when suspension was dropped into the conjunctival sac without injection. However, tumor take has been noted to occur first on the posterior surface of the avascular cornea in four eyes in the Greene series. Injection of the tumor suspension into the choroid results in a malignant melanoma of the choroid (fig. 30).

The viability of the tumor cells was illustrated by obtaining takes in two of four eyes injected with 0.0025 ml. of 1:10 dilution of tumor suspension stored at refrigerator temperature for seven days before injection into the hamster.

In one day's group of 18 animals, when the Hamilton microsyringe was sterilized by ethylene oxide⁸ rather than heat, all in-

jected eyes developed a panophthalmitis. When the animals were first examined two weeks later, *Sarcina* were cultured. No ocular growth of tumor was noted in this entire series, illustrating a possible deleterious effect of acute infection on tumor take. That the tumor suspension contained viable cells was shown by the success of two subcutaneous transplants done with the same syringe from the same tumor suspension preparation.

DISCUSSION

The Greene tumor produces malignant melanoma of the iris which spreads over the iris, blocks the chamber angle and closely simulates the syndrome of glaucoma due to diffuse melanoma of the iris in humans. In contrast, the Fortner tumor produces more localized growth which resembles the commoner, less malignant, less likely to spread, iris melanoma. With the Greene tumor the type of growth varies with the amount of injection. With higher concentration of tumor, a diffuse growth of tumor over the iris occurs, while with weak dilutions solitary tumors are seen.

We feel that this technique is an excellent tool for study of malignant melanoma of the eye, since so many parameters of human malignant melanoma occur: variable amount

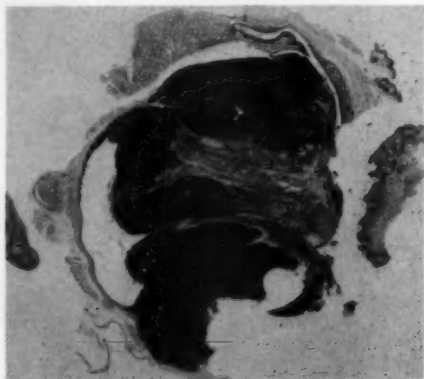


Fig. 29 (Burns and Fraunfelder). Fortner tumor of eye which had become infected and necrotic but still fairly well confined within eye at 142 days. Hematoxylin-eosin, magnified 15 \times .

of pigment, variable rate of local growth, variable speed of metastasis, delay of onset of tumor growth after inoculation and delay in appearance of metastases after enucleation. Since human malignant melanoma is such a protracted disease, with iris and choroidal melanomas existing for many years and metastases notorious for their late appearance, it is fortunate that an experimental technique can so closely simulate the variability in malignancy and delay in metastasis of the human counterpart. Much has been learned in human genetics by the study of the *Drosophila* and *Escherichia coli* bacteriophages; it is hoped to understand human malignant melanoma better by an inexpensive animal model that can be studied in a few months.

This work is only in its preliminary stages. The cell suspension technique was used in early anterior chamber transplants but has been abandoned recently in favor of solid blocks of tissue. It is felt that use of cell suspensions are of great value since different clinical pictures can be simulated by different amounts and dilutions of injection; it is less traumatic and more accurate than injection of a solid mass of tumor via a trocar. It is hoped that even more accuracy in inoculation can be obtained with finer dispersal of the cell solution by mechanical or enzymatic means so that accurate cell counts can be obtained. It is planned to use single cells, isolated by micromanipulation techniques, for inoculation.

In addition, if antibodies can be produced by injection of these melanomas it may lead to the possibility of a new treatment of malignant melanoma and the effect of hypothermia on tumor growth is to be studied. Visible growth in the anterior chamber of the eye is an excellent site for assaying results of treatment.

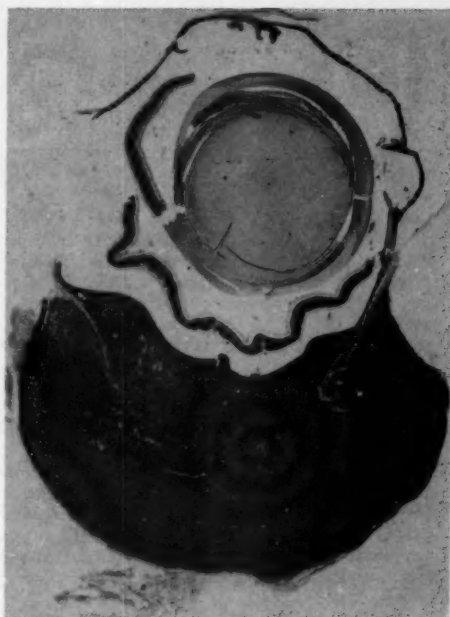


Fig. 30 (Burns and Fraunfelder). Malignant melanoma (Greene type) of choroid, produced by injecting cell suspension into choroid. Hematoxylin-eosin, magnified 15 \times .

SUMMARY

1. A technique for production of experimental malignant melanoma of the iris in Syrian Golden hamsters with two different strains of malignant melanoma is described.
 2. A description of the variable effects of the different malignant melanomas is given.
 3. Early studies indicate that enucleation of a melanoma-bearing eye is a valuable therapeutic measure.
 4. The similarity of the human and experimental malignant melanoma and the value of the experimental approach to clinical study of human neoplasia is pointed out.
- University of Oregon Medical School
(1).

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DISCUSSION

DR. JOHN S. MCGAVIC [Bryn Mawr, Pennsylvania]: Dr. Burns and Dr. Fraunfelder have presented an excellent preliminary report of their experiences in growing malignant melanomas in the eyes of animals. I personally have never come to grips with the Syrian Golden hamster, so I am not quite equipped to discuss this paper adequately. I was unaware that these animals developed spontaneous malignant melanomas in the skin.

I have followed some of the work of Dr. H. S. N. Greene in transplanting tumors, and I feel he has made a tremendous contribution to oncology. We have a lot to learn about various types of melanomas, and I am glad now that more ophthalmologists are working in this field, particularly because our viewpoint is necessarily different from that of the general surgeon and the general pathologists who deal with cutaneous melanomas almost exclusively in human subjects.

I would like to ask the essayists whether the original cutaneous tumors behave as differently in the hamster clinically as they do in the authors' experimental injections.

In the human subject, malignant melanomas of the skin and those of the uveal tract behave much differently, as you know. Those of the skin and conjunctiva, particularly in precancerous and cancerous melanosis in the ocular adnexa, are more malignant and spread by lymphatics and also metastasize by lymphatics. Those in the uveal tract metastasize by the blood stream. The difference in prognosis is also quite definite. In general, those arising from the cancerous melanosis are more rapidly lethal.

The reason for the long delay in distal metastases of those tumors of the uveal tract has kept everyone baffled up to date. We hope Dr. Burns and Dr. Fraunfelder will be able to elucidate this phenomenon some time later.

The difference in the manner of growth of the Greene and Fortner type of melanomas is quite remarkable, and it is interesting that they find the similarities they do with the uveal tract melanomas in the human eye. The authors realize, I am sure (although they did not say so), the impossibility of drawing too definite conclusions from animal tumors, especially those from the skin, as compared with uveal melanomas in the human eye.

In this regard, I think we should encourage the essayists to go on with their work, and particularly to work with the human malignant melanomas as soon as they can and as much as possible. We all know that the heterologous transplants are much more difficult to produce as compared with the homologous. As far as I know, no one has grown a human malignant melanoma from the uveal tract

in the anterior chamber of an animal eye.

I would suggest that the authors try first growing the human tumors in tissue culture and then transplant them into the hamster eye. I think this has been tried unsuccessfully in rabbit eyes. Whether or not it can be done, I don't know.

If they then follow the tissue culture by the technique they worked out so nicely, they might be successful in getting growth of human tissue.

Dr. Reese and others of his colleagues have grown malignant melanomas in tissue cultures, and they feel that they can determine by the manner of growth whether the tumor arose from the skin or from the uveal tract—that is, whether it arose from a junctional nevus or from cancerous melanosis or from the uveal tract by the way the tissue culture growth goes.

There probably would be some difference in the growth in the hamster if the tissues are first cultured and then transplanted. I know this is asking a good bit, to suggest to someone who just started out on the problem, but we hope they will continue.

I will look forward to the results of studying the reasons for the difference in the behavior of the malignant tumors of the iris and those in the choroid in the future. It would be nice to find out also why melanocytomas as described by Zimmerman in the nerve head are so benign. The essayists are to be congratulated on their very careful work. Their report is excellent. We hope nothing deters them from studying the problems they have listed in the paper and studying malignant melanomas in the human eye.

DR. ROBERT P. BURNS (Closing): First, the tumors we inoculated into the hamster eye behave quite similarly to the way they do when transplanted subcutaneously with one difference. We obtain these in subcutaneous transplants and were told that the Fortner tumor, which is one of three that Dr. Fortner described to us, and which was the one that seemed to be best for our work, would take only in males and not in females. We found it took equally well in both male and female eyes.

There are an amazing number of clinical similarities in these hamsters and in humans. First, one must remember that the life span of a hamster is two to three years; so if one obtains a cure of three months in a hamster it may be equivalent to around a five to ten-year cure in a human.

The reason why we reported such a small series of enucleated animals is that we did want to keep our survival times this long. We have a great number more that have not survived as long as these, that are under observation.

There is another resemblance in these hamsters

whose malignant melanomas have been enucleated, which correlates with the human problem, namely, that they may show up with delayed metastases quite a while after the eye has been enucleated. Rather than pursuing a steady downhill course, they seem to be healthy for a while and then develop extensive metastases.

We have injected the tumor in the eye of several animals and nothing may appear for one or two months or even more, and then suddenly the tumor will appear and will grow at its ordinary rate. Whether this is due to an antigen-antibody relationship, we do not know. We hope to find out in the future.

As far as growing these particular tumors or human tumors in tissue cultures and then injecting them is concerned, that would be open to the objection that human normal tissues after grown in serial passage tissue culture take on an anaerobic form of glucose metabolism, and there are a number of other differences that have been demonstrated between tissue culture-carried normal cells and the cell when it is first isolated. So, that would introduce a certain variation into the experiment, as well as the problem of a heterologous transplant.

We plan to continue this work. May I thank Dr. McGavic for his kind encouragement.

INFLUENCE OF AGE AND CATARACT FORMATION ON THE RIBONUCLEIC ACID OF THE LENS

I. CHANGES IN VARIOUS FRACTIONS OF RIBONUCLEIC ACID OF RAT LENSES DURING AGING*

ZACHARIAS DISCHE, M.D., ANIMA DEVI, PH.D. AND GINEVRA ZELMENIS, PH.D.

New York City

Evidence has been previously set forth^{1,2,3} that in various forms of experimental cataracts of rat there is already in the initial stage of this pathologic process a significant more or less pronounced inhibition of the net synthesis of proteins. Experimental cataracts can be much easier produced in young rats in which the rate of net synthesis of lens proteins is very high than in mature animals. Investigations of the last few years have demonstrated the important role which ribonucleic acids (RNA) play in the synthesis of cellular proteins. It has been made probable that this synthesis proceeds at least in two successive steps, in each of which a special form of RNA plays a decisive role. In the first stage activated amino acids combine with the low molecular RNA which is not bound to particles and cannot be spun down even after three hours by a centrifugal force of 105,000 G. This is the so-called soluble RNA (SRNA). This combination of amino acids and SRNA is then transferred to RNA present in the so-called ribo-

somes, a particle fraction, inhomogeneous as to their size, coming successively down from homogenates of various cells at various speeds of centrifugation up to 105,000 G. At this second stage the specific chain of amino acids characteristic for every protein is supposed to be synthesized. In addition, one RNA fraction is always found also in mitochondrial particles. The significance of this fraction so far has not been clarified.

It seemed possible that changes in the net synthesis of lens protein observed during the cataractous processes as well as during the aging of the lens may either accompany or follow changes of RNA as a whole, or its particular fractions. Experiments, therefore, have been started in which first the content and distribution of RNA in rat lenses of various ages were investigated, as well as their changes in experimental cataracts. The present report deals with the first part of these studies, namely the influence of the age of the animal on the concentration and distribution of RNA in lenses of rats.[†]

* From the Department of Ophthalmology, College of Physicians and Surgeons. This work was supported by grant B 1192 of the National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service. Read at the Miami Meeting, June 1960.

† Surprisingly little has been reported on the RNA of the lens. The only relevant publication is a short note by Mandel and Schmitt in 1949⁴ who determined RNA in beef lenses. They found on the average only between 42 and 47 mg RNA/100 gms lens material, which is about 10-20 times less than

EXPERIMENTAL

1. ANIMAL MATERIAL

Four week to 16-month-old rats were commercial animals kept on standard laboratory diet. Three kinds of senile animals from the Columbia old age rat colony* were used. (1) 735 to 900 day old males which lived under normal conditions and were killed or died from various spontaneous diseases. (2) Rats kept under various conditions of stress. (3) Very old females 1046 to 1180 days of age which were killed at this age and were kept on a calorically restricted diet, which depressed the final body weight by about 25 to 40 percent. Some groups of these lenses were kept frozen one to two months before being used for analysis. A control experiment with left lenses of one-year-old rats kept frozen for three weeks did not show significant changes in distribution or content of RNA as compared with the right lenses of the same animals used immediately after killing.

2. PREPARATION AND ANALYTICAL PROCEDURES

For each determination 200 to 400 mg. of lenses removed from rats killed with ether were dried with filter paper and weighed. They were homogenized in a 10-fold amount of ice cold distilled water.

a. *Fractionation of lens homogenates.* The homogenates were subjected to fractionated centrifugation in a Spinco preparative ultracentrifuge. As homogenization in distilled water appeared advantageous for further

chemical subfractionation of the soluble fraction in two control experiments, the homogenization and fractionation were simultaneously carried out on 10 right lenses with distilled water and on 10 left lenses with a mixture of either four parts isotonic KCl and one part of 0.1 M Na_2HPO_4 solution of pH 8.2 or M/150 0.1 M solution of Na_2HPO_4 pH 8.2.⁴ It was found that a certain shift was noticeable in the distribution of individual fractions in the sense that somewhat more RNA appeared as a soluble fraction in the buffer homogenates than in H_2O homogenates. As in our investigation the interest concentrated on the relative content in SRNA and the particulate RNA fractions, we chose the homogenization with H_2O as a standard procedure as obviously the 12 to 15 percent lower values of the soluble fraction in H_2O homogenates appeared more closely to correspond to SRNA than the somewhat higher values in buffer homogenates. In general, the first fraction was obtained by spinning down the homogenate for one hour at 12,000 RPM (10,000 G). This fraction (12,000) was later shown to consist of a main subfraction sedimenting at 600 G in 30 minutes and a much smaller fraction coming down at 12,000 RPM in one hour, which probably represents a mixture of mitochondria and microsomes. In four experiments these two subfractions have been separated. The sediment was first washed twice with distilled water, once with saline and finally with 5 percent TCA. The supernatant diluted with H_2O to 8.0 ml., to obtain a sufficient volume, was then centrifuged for two hours at 30,000 RPM. Longer centrifugation at this speed did not yield more sediment. Centrifugation at 40,000 RPM for three hours followed, the supernatant fluid being stirred vigorously after each hour to assure that as large a proportion of the particles be exposed to the maximum speed of 144,000 G. The sediments from these two fractions were washed once with 8 to 9 ml. H_2O . The final supernatant was precipitated

what is found in parenchymal tissues, like liver, kidney and pancreas. In a second investigation Mandel and assoc.^{4a} determined RNA separately in a thin anterior subcapsular, in the cortical and nuclear parts of the calf lens and reported that the last one contains only one-fifth RNA in per cent of protein as the second, and one-tenth as the first subcapsular part of the lens.

* We are greatly indebted to Dr. Benjamin Berg of the College of Physicians and Surgeons, Columbia University, who greatly facilitated this investigation by providing lenses of two- to three-year-old rats from his colony.

and twice washed with 5 percent TCA.

b. *Determination of RNA and N.* RNA was extracted from the TCA precipitates either by the procedure of Schneider,⁵ with omission of the lipid extraction which was shown to be unnecessary in the case of the lens, or with 10 percent HClO_4 at 4.0°C according to Ogur and Rosen.⁶ For the complete extraction with HClO_4 it was found necessary to incubate for 96 hours. 24 hours extraction yielded only about 80 to 90 percent of the total RNA. The determination of RNA was carried out routinely by Bial's orcinol reaction according to Dische and Schwartz.^{7*} That the extracted pentose is present in form of RNA was ascertained first by the rate of development of color in the orcinol reaction when compared with a standard of pure RNA and then by determining in a few cases RNA in the 12,000 fraction by the orcinol reaction, as well as two other reactions, namely the phloroglucinol reaction⁸ and the cysteine- H_2SO_4 reaction¹⁰ of pentoses which are both based on completely different principles than the orcinol reaction and in which pentoses substituted in position five and three significantly differ in their extinction coefficients. Identical values obtained by these additional reactions, therefore, permit to conclude that ribose is not substituted in position five. It has not been possible so far to determine RNA in 30,000, 40,000 and SRNA fractions by other color reactions than the orcinol reaction, because of low concentrations of RNA in the extracts of these fractions. The presence of pyrimidine nucleotides, on the other hand, which do not react significantly in the mentioned color reaction, was ascertained by comparing the differences of optical densities at 260 $\text{m}\mu$ and 250 $\text{m}\mu$ in

HClO_4 extracted solutions of lens RNA fractions with that of a standard of pure yeast RNA. The absorption curves between 250 and 278 $\text{m}\mu$ for the nuclear RNA and fractions 3,000 and 30,000 from one-year-old rats follow closely the absorption curve of yeast RNA (figs. I and II). The difference of optical densities at 260 and 250 $\text{m}\mu$ for these preparations is in satisfactory agreement with the amount of RNA calculated from the orcinol reaction.[†] The absorption curve for SRNA on the other hand, agrees with the others in the position of the maximum, but deviates significantly in its course. The designation of this fraction as soluble RNA, based on the absorption curve in the orcinol reaction, extractability conditions with TCA and HClO_4 and precipitability at pH 5.0 is at present very tentative. An attempt to purify this fraction and further identify it is under way (fig. I). Nitrogen was determined by micro-Kjeldahl in mixtures of equal volumes of TCA extracts and extraction residues, dissolved in the same volume of 0.5 N NaOH as that of TCA used for the extraction.

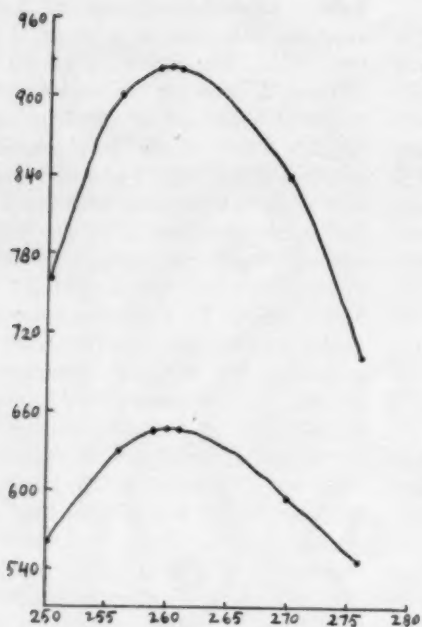
RESULTS

1. CHANGES WITH AGING IN THE CONCENTRATION OF RNA IN THE LENS AS A WHOLE

RNA first has been determined in whole lenses of rats in three different age classes: (1) four to 10-week-old males and females, (2) sexually mature 11 week to 16-month-old rats of both sexes and (3) two groups of old rats, one 800 to 900 days old males which died of natural causes, the other females between 1140-1180 days of age kept on a restricted diet. (The dietary restriction resulted in a slow-down of the growth rate of about 10 percent as measured by tibial

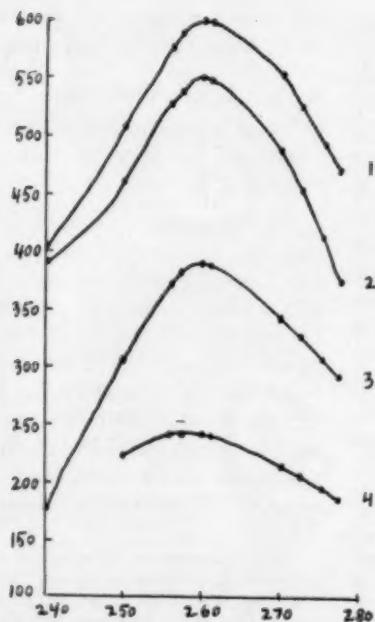
* In the orcinol reaction dichromatic measurements of optical densities at 670 degrees and 580 $\text{m}\mu$ were made in the Beckman Spectrophotometer; under these conditions it was found that DNA of the lens, which was determined by Burton's modification of the diphenylamine reaction⁹ had a negligible effect on RNA determinations even in the separated equatorial part of the lens.

† The discrepancies between the spectra of lens and yeast RNA between 260 and 250 $\text{m}\mu$ have been found to be due to impurities in TCA used for washing of RNA fractions and not totally eliminated during subsequent washing with 10 per cent HClO_4 .



ABSORPTION CURVE OF
YEAST RNA 35 $\mu\text{g}/\text{ML}$

ABSORPTION CURVE OF
NUCLEAR HClO_4 EXTRACT 18 $\mu\text{g}/\text{ML}$ RNA



ABSORPTION CURVES IN 2.5% HClO_4

2 RNA 17.5 $\mu\text{g}/\text{ML}$

1 3,000 FR. CORR. 16 $\mu\text{g}/\text{ML}$ RNA

3 30,000 FR.

4 SRNA

length and 25 to 40 percent based on body weight.) The results of these determinations are listed in Table I. As can be seen, there is a continuous decline in the concentration of RNA during the life span, whether calculated per wet weight or amount of protein. From 250 to 280 $\mu\text{g. RNA per } 100 \text{ mg. lens}$ in six-week-old rats these values go down by more than one-third during the first year of life and come down to about one-third in the very old rats.

A different picture, however, appears when the content of RNA is calculated per individual lens. On this basis, the value for RNA rises from 54 and 78 $\mu\text{g./lens}$ at six

weeks to 95 to 112 $\mu\text{g./lens}$ in 12 to 16-month-old rats but appears again at a significantly lower level where the values drop again to about 50 $\mu\text{g./lens}$ in the very old rats.

2. DISTRIBUTION OF RNA AMONG VARIOUS CYTOPLASMIC FRACTIONS AT DIFFERENT AGES

The fractionation of the cytoplasmic RNA has been carried out on the same three classes of animals as were used in the first experimental series, except that among the very old rats in addition to the two previously mentioned groups, a third group was

TABLE I
TOTAL RNA CONTENT OF RAT LENSES AT VARIOUS AGES

	Exp. No.	Number of Lenses	Net Weight	Age	RNA $\mu\text{g./lens}$	RNA $\mu\text{g./}100 \text{ mg. Lens}$	RNA $\mu\text{g./}100 \text{ mg. Protein}$	Remarks
1	87a	10	19.1	28 days	52.2	275	690	Female
	87b	10	23.5	33 days	67.0	285	681	Female
	87c	10	25.9	38 days	78.5	303	745	Female
2	44a	5	24.8	41 days	63.2	241		Male
	44b	5	25.4	45 days	63.8	245	613	Male
3	1	9	24.3	41 days	67.2	275		Male
4		9	25.4	41 days	70.5	274		Male
5	80	4	25.3	56 days	53.4	210	528	Male
6		4	27.9	56 days	55.1	208	532	Female
7	93a	12	36.0	10 weeks	112.0	292		Male; HC10, extracted
	93b	4	39.3	12 weeks	98.7	252	691	Male
	93c	8	50.2	16 weeks	90.0	180	561	Male
8	62	8	54.0	About 1 year	100.3	191	441	Male
9		8	55.5	About 1 year	101.3	187	429	Male
10	67a	7	61.3	About 1 year	109.5	149	413.5	Male
	67b	7	60.1	About 1 year	111.0	154	415.5	Male
11		12	58.5	About 15 months	117	187	439	Female
12	64	4	63.7	765 days	72.0	108	243	Male; normal diet
13	63	4	60.0	735 days	76.8	128	254	Male; normal diet
14	103	4	60.0	880 days (average)	49.0	79.5	238.5	Male; normal diet
15	36	12	58.0	1,173 days	35.7	61.5		Female; restricted diet
16	106	4	58.0	1,142 days	69.0	106		Female; restricted diet

TABLE II
DISTRIBUTION OF RNA AMONG VARIOUS PARTICULATE AND THE SOLUBLE FRACTIONS
IN LENSES OF RATS AT VARIOUS AGES

Exp. No.	Age	Number of Lenses	Net Weight mg./ Lens	Fractions								Remarks
				12,000		30,000		40,000		SRNA		
				µg./ Lens	% Prot.	µg./ Lens	% Prot.	µg./ Lens	% Prot.	µg./ Lens	% Prot.	
44	41 days	16	25.4	31.9	1.50	10.3	5.7	1.1	1.65	10.0	0.12	Male
	46 days	16	27.0	37.2	1.60	11.0	4.6	5.5	2.7	11.6	0.15	Male
	46 days	8	26.5	17.8		11.7		1.7				
40	42 days	15	21.8	35.1	2.40	11.5	4.4	0.95	0.45	10.65	0.15	Male
33	77 days	8	35.4	61.9	1.80							Male
38	4 months	10	41.9	61.2	1.05	8.9	1.4	0.65	0.4	12.0	0.09	Male
43	About 1 year	7	56.6	67.5	0.65	10.3	2.5	2.35	0.55	18.6	0.13	Male
		7	57.5	72.8	0.85	5.7	2.0	1.15	1.45	16.9	0.14	Male
111	About 16 months	12	62.0	64.9	0.70							Female
46A	901 days	6	59.9	25.6	0.16	2.5	0.50	1.1	0.40	8.6	0.11	Male stressed
119a	886 days	4	62.0	51.8	0.34	3.2	1.6	1.1	1.05	7.6	0.11	Male stressed
119b	902 days	4	58.0	42.5	0.24	2.4	3.7	1.1	1.20	6.3	0.11	Male stressed
42	1,164 days	8	58.0	33.6	0.39	0.7	0.22	0.7	1.20	10.4	0.11	Female restricted diet
46	1,178 days	6	54.0	29.5	0.22	3.1	0.40	1.1	0.45	5.0	0.08	Female restricted diet

represented which consisted of animals exposed during their life time to various forms of stress. Of 14 groups of experimental rats in four representing all three age classes, the 12,000 fraction was subfractionated into fractions 3,000 and 3,000 to 12,000. The results of the four experiments are found in Table III. The results of the second series of experiments in which fraction 12,000 was not subfractionated are listed in Table II. As can be seen from Table III, at 3,000 RPM most of the particulate RNA already sediments in 10 minutes. Further centrifugation at this speed for another 20-minute period increases the yield of this fraction by no more than two to three percent. Both these fractions may be, therefore, associated with the albuminoid itself. They will be designated as albuminoid RNA. A sizeable fraction sediments after one hour centrifugation at 12,000 RPM (3,000 to 12,000 fraction). The 3,000 to 12,000 fraction with the highest RNA content of all probably corresponds to a mixture of mitochondrial and microsomal RNA, whereas the 30,000 fraction can be re-

garded as purely microsomal. The small 40,000 fraction contains significant amounts of protein, but the content in RNA was at the limit of error of the method of determination. Its significance, therefore, is not clear.* The fractions 30,000 and 40,000 will be referred to as small particle RNA. All three fractions of particulate RNA show changes with the age. These changes with age, however, differ from one fraction to another as far as direction and degree of the changes are concerned. Thus, during the first year of life the 3,000 fraction increases considerably though not to the same extent as the corresponding protein. The 30,000 fraction changes only slightly, whereas the 3,000 to 12,000 fraction sharply decreases. In the very old rats, which were investigated in this experimental series, there is also a sharp decrease in the 30,000 fraction. But still much more pronounced than the decline in the absolute amounts of the small particle RNA in the individual lens, appears the

*It may represent the endoplasmic reticulum which with age lost most or all of attached ribosomes.

continuous decrease in the ratio RNA to protein in the 3,000 to 12,000 and 30,000 fractions with the age, which in the very old group drops to less than one-fifth of that in six-week-old rats.

The soluble RNA represents about 20 percent of the total RNA in the young as well as in older rats. Consequently, this fraction sharply increases during the first year of life so that up to about 20 μ g. of SRNA/lens are found in one-year-old rats as opposed to approximately 10 μ g. in lenses of young animals. Here again in very old rats there is a marked decrease in the amount of this fraction per lens. This, however, does not reach the high proportions observed in the 30,000 fraction particularly when the ratio RNA to protein is considered.

3. PRECIPITATION OF SRNA WITH ONE PART OF ALPHA CRYSTALLINE

SRNA found in other living cells was shown to be precipitable at a pH around 5.0. As under these conditions alpha crystalline also becomes insoluble, it was to be expected that SRNA would be obtained in combination with alpha crystalline. To test this the supernatant of the complete fractionation of lens homogenates, in which SRNA had been previously determined, were adjusted with diluted acetic acid to a pH 5.2, the precipitate of alpha crystalline was separated by centrifugation and the supernatant dialyzed for 24 hours against large volumes

of distilled water (pH about 5), renewed several times a day. A second fraction of alpha crystalline separates under these conditions. These two alpha crystalline fractions were washed with distilled water and five percent TCA. The final supernatant was then precipitated and the precipitate washed with five percent TCA free of nucleotides. RNA and N was then determined in all precipitates.

About 86 percent of the SRNA was found in the first alpha crystalline precipitate which contained only one-fourth to one-third of the total alpha crystalline. Beta crystalline was found free of RNA.

4. DISTRIBUTION OF RNA AMONG DIFFERENT REGIONS OF THE LENS

To determine whether the RNA content parallels the rate of protein synthesis in different regions of the lens, a series of experiments was carried out in which lenses were sectioned in four or five parts with appropriate trephining operations. Lenses frozen immediately after extraction were put on a glass plate with their anterior posterior axis perpendicular to the surface of the glass plate and an equatorial ring was cut out (equatorial part). A second section parallel to the first is here designated as the sub-equatorial part. The lens was then tilted 90 degrees and a narrow band was cut out containing the polar region. The remaining portion of the lens representing 40 to 60 per-

TABLE III
SUBFRACTIONATION OF THE 12,000 RPM FRACTION OF LENS RNA AT DIFFERENT AGES

Exp. No.	Age	Number of Lenses	Fraction											
			3,000 RPM 10 minutes				3,000 RPM 20 minutes				12,000 RPM 1 hour			
			1	2	3	4	1	2	3	4	1	2	3	4
I	45 days	8									43.2	15.0	17.8	7,772
II	About one year	13	81.0	93.8	40.5	638	1.5	1.7	0.5	623	17.5	4.5	8.6	2,726
III	902 days	4	96.0	95.6	41.0	234	4.0	2.4	1.7	420	4.5	2.0	2.0	560
IV	886 days	4	90.0	93.6	49.3	328	2.5	2.8	1.2	327	7.5	3.6	4.2	720

1 = RNA in percent of total RNA of 12,000 fraction.

2 = Protein in percent of total protein of 12,000 fraction.

3 = RNA μ g./lens in subfraction.

4 = RNA μ g./100 mg. protein in subfraction.

cent of the total weight of the lens is designated as the total nuclear part. In some experiments this part was subdivided in a more peripheral and more central part of about equal weight. RNA and proteins in every part of the lens were then determined and their ratio calculated. The results are listed in Table IV. In lenses of four to six-week-old rats, the RNA per 100 mg. protein is very significantly smaller in the nuclear than in the equatorial part. The high content in the equatorial part, however, drops very sharply during the first year of life by 50 to 70 percent. A further decrease by about 25 to 50 percent in the equatorial part is observed in the very old group. Changes in the nuclear part calculated on protein basis between six weeks and one year are insignificant, and the most central parts of the nucleus show a higher content of RNA than the more peripheral parts.

DISCUSSION

We find in the lens RNA in three forms: (1) RNA linked to the albuminoid, (2) RNA in particles which on the basis of their sedimentation rate in the ultracentrifuge and their content in RNA in the case of young rats correspond to the microsomal and probably also mitochondrial fraction and (3) nonparticulate "soluble" RNA precipitating at pH 5.0 which seems to correspond to SRNA found in other living cells. No RNA fraction analogous to that linked to albuminoid seems to have been found so far in other animal tissues. This may be due to the fact that albuminoid which apparently is located on the surface of the subcortical lens fibers represents a cellular element not homologous to any so far isolated in larger quantities from other cells of higher animals. In certain bacteria, however, like *staphylococcus aureus*,¹¹ *azotobacter*¹² and *alkaligenes faecalis*,¹³ it was found that the cellular membrane which can catalyze protein synthesis in absence of any ribosomal elements contains RNA in amounts comparable to those

found in albuminoid.*

Aging of the lens changes its content in RNA as well as the distribution of the latter among its various fractions. The amount of RNA per unit volume or protein decreases continuously with the aging of the animal. The fact that the amount of RNA in the individual lens rises during the first three to four months of life from 54 to 78 $\mu\text{g.}$ to 90 to 100 $\mu\text{g.}$ and then remains almost constant during the first year does not at all indicate that aging of lens fibers does not affect the total amount of RNA of the individual fiber. This becomes clear when the characteristic growth pattern of the lens is considered. Growth in the lens during the whole life span goes on in such a way that newly formed lens fibers are laid down on the surface of those present at a certain stage pushing the latter continuously toward the center of the lens. A lens of a three-month-old rat weighs between 25 and 30 mg. and contains about 100 $\mu\text{g.}$ of RNA. During the life span all the material present in the three-month-old rat will be shifted toward the center of the lens. In a one-year-old rat the whole lens contains the same amount of RNA as a three-month-old rat but the material which at the latter age represented all of the lens is now concentrated in the central part of the lens of the one year old rat, which corresponds by weight to about half of the whole lens of the one-year-old rat. This part of the lens obviously contains only a fraction of the RNA present in the whole lens of the one-year-old rat.

It is clear, therefore, that fibers present in the three to four-month-old lens lose a part of their RNA in the following period of about one year. Aging of lens fibers, therefore, is associated even during the first year of life with a continuous depletion in RNA. This indicates that the continued loss

* An analogous RNA fraction may be represented by the cortical RNA of the maturing amphibian oocyte which disappears when the synthesis of yolk proteins stops.

TABLE IV
TOPICAL DISTRIBUTION OF RNA AMONG DIFFERENT PARTS OF THE RAT LENS AT VARIOUS AGES

Exp. No.	Age	No. of Lenses	Parts of Lens															Remarks			
			Equatorial			Subequatorial			Polar			Peripheral Nuclear			Central Nuclear				Total Nuclear		
			1	2	3	1	2	3	1	2	3	1	2	3	1	2	3		1	2	3
93	4 weeks	19	21.0	264	765	26.5	152	562	23.0	227	435				29.5	180	292	Normal condit. Normal condit.			
40	6 weeks	16	20.0	430	990				8.0	217	801				72.0	294	383				
94	3 months	14	20.0	154	492	16.0	134	395	21.0	153	290				43.0	262	334				
109	About 1 year	20	28.0	156	366	5.0	197	288	8.0	150	369				59.0	274*	394*				
112	About 1 year	9	31.0	131	355				13.5	149	444				55.0	207*	349*				
99	About 1 year	8	24.5	133	449	6.0	120	309	13.0	165	377				56.5	226	380				
96	15 months	8	28.0	189	536	6.7	149	337	7.7	162	335				57.6	293	437				
103	798 days	4	29.0	66	232				8.0	105	351				63.0	109	235				
104	918 days	4	19.0	111	280				8.5	135	324				72.0	627	251				

1 = weight of parts in percent of weight of whole lens.

2 = $\mu\text{g.}/100 \text{ mg. lens material.}$

3 = $\mu\text{g.}/100 \text{ mg. protein.}$

* Calculated as sum of peripheral and central nuclear.

of RNA by the individual lens in senility is at least partly due to the aging itself and becomes apparent as soon as the RNA which leaks out of the fibers cannot be reutilized in rapidly growing new lens fibers. Our experimental material, however, does not permit a definite conclusion as to how much pathologic processes and other conditions of the senile animals contribute to the loss of RNA.

This loss appears to be mainly due to a sharp decrease in the RNA/protein ratio of the small cytoplasmic particles. This decrease appears to be the most striking change in RNA in senility and can be easily correlated with an almost complete disappearance of net protein synthesis. Decreases in albuminoid RNA and SRNA calculated per protein unit appear to be less pronounced and do not parallel decrease in net protein synthesis. This appears particularly true of SRNA. These two fractions may be, however, related to the synthesis of albuminoid which continues during the whole life span. It seems also possible that RNA in these two fractions in some way acts as a highly polarized polyelectrolyte with great affinity to lens proteins. Such a polyelectrolyte by its electric charges could have great influence on the physicochemical state of lens proteins. This possibility is also suggested by the lesser rate of extraction of RNA by HClO_4 , particularly from the albuminoid and the relative constancy of the SRNA/protein ratio during the life span.

The small, if any, difference between the concentration of RNA within the nuclear and cortical parts of the lens of the mature rat can be explained by the fact that the albuminoid in a one-year-old rat is mostly concentrated in the nuclear part. Separate fractionation of the nuclear and the combined three cortical sections of lenses of one-year-old rats showed that most of the RNA of the nuclear part was present in form of

the albuminoid linked RNA, whereas the two other fractions represented only a small fraction of those found in the cortical parts. The albuminoid RNA, on the other hand, was almost nonexistent in the cortical parts. This appears to be in agreement with the fact that the albuminoid but not soluble protein, is continuously synthesized in the nuclear part of the lens during the whole life span.

SUMMARY

1. Rat lenses contain polynucleotides attached to protein which, on the basis of the absorption in the ultraviolet and in several color reactions of pentoses, appear closely related to ribonucleic acids obtained from other sources.
2. The lenticular RNA appears in three different forms. (1) As microsomal and possibly mitochondrial RNA, (2) soluble RNA, and (3) RNA linked to albuminoid. The latter even in young animals represents more than 50 per cent of the total RNA.
3. The nuclear part of the rat lens contains about as much RNA as the cortical. But in the first most of it is linked to the albuminoid and very little to other fractions.
4. The concentration of RNA calculated per 100 mg. wet weight, or per 100 mg. protein, declines continuously during the life span.
5. The amount of RNA in the individual lens reaches almost the maximum at about three months and little, if at all, changes during the following one year period.
6. The microsomes of the lens suffer a continuous strong depletion in RNA during the whole life span, although the amount of microsomal protein present in the individual lens does not significantly vary with age.
7. Data are discussed which indicate a continuous depletion in RNA of individual lens fibers during the whole life span of the animal.

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DISCUSSION

DR. JOHN F. R. KUCK, JR. [Detroit, Michigan]: This paper is one of a series dealing with protein metabolism in the rat lens preceding experimental cataract formation. The decreased protein synthesis shown previously in the aging lens should logically be accompanied by changes in RNA concentration, and this study shows that this is indeed the case.

The continuous decline in RNA concentration with age is to be expected as a reflection of the decelerating growth rate of the lens.

The occurrence of a peak value in total RNA content at age one year appears to be correlated with a marked deceleration of growth and decreased protein turnover rate, with the result that a fully mature lens has no more RNA than a very young, growing lens.

The RNA concentration in different sections of the lens is unequal, the higher levels being found at the equator and in the nucleus. This unusual distribution Dr. Dische finds is due partly to the fact that RNA in the nucleus is strongly bound to albuminoid, and here the albuminoid is the major part of the lens protein.

The high RNA level at the equator is of course to be expected, since here active cell multiplication is occurring. These results form an excellent basis for

the development of Dr. Dische's thesis that disturbed protein metabolism occurs very early in the cataractous process. It will be most gratifying if he is able to show that this metabolic disturbance is a common factor in several forms of cataract.

I would like to ask the essayist if the relatively high RNA level in the nucleus implies that protein metabolism is more active here than in the sub-equatorial region, for instance.

DR. LUDWIG VON SALLMANN (Bethesda, Maryland): I would like to ask Dr. Dische whether he has identified electron-microscopically the various fractions which he obtained by high-speed centrifugation. If this was done, could he show that in the last fraction the microsomes were the prevalent element.

DR. ZACHARIAS DISCHE (closing): To answer the question by Dr. Kuck, I would like to point out again that we really know very little about the turnover of the lens proteins, particularly in the nuclear part in the rat lens, and it is quite possible that this turnover may be very significant in the nuclear part and requires the presence of considerable amounts of RNA.

However, we must also consider that there is a very pronounced net synthesis of albuminoid in the

nuclear part, and the RNA in the nucleus is mostly the RNA of the 3,000 r.p.m. fraction. That means it is bound to albuminoid. It is clear that we cannot extract from the albuminoid the RNA fraction in 24 hours with 10 percent perchloric acid as is easily done in the liver or kidney. It takes about 96 hours to make the extraction complete, which certainly indicates a specific link that has nothing to do with the concentration of the protein because the soluble RNA is much easier extracted, although it is lower than in the albuminoid fraction.

We must consider the possibility that the RNA has some influence either on the production of albuminoid itself or on the physical-chemical state of the albuminoid and possibly other lens proteins as a strong polyelectrolyte that has a high affinity to pro-

teins and may change the size of the molecule and the asymmetry of the molecule.

Concerning Dr. Von Sallmann's question, I must state with regret that we did not identify by direct electromicroscopy and microscopy that we are dealing here really with microsomes. We rely in our nomenclature on what is usually done in the literature. In general it is assumed that the fractions which come down, at 40,000 r.p.m. up to 105,000 G are ribosomes, so we accepted that, but we really did not prove that by direct microscopy. For this purpose we would have not to do the homogenates in distilled water but at least in isotonic salt solution to preserve the integrity of the material. We intend to do that now in a continuation of this work.

THE OCCURRENCE OF PROTEASES IN BOVINE LENS

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In recent years several studies of the hydrolysis of proteins by proteolytic enzymes of various tissues have led to the discovery of more than one pH optimum, a finding generally interpreted as indicating the presence of more than one protease. The occurrence of a number of tissue enzymes acting under acid conditions has also been recognized. Ball¹ described the purification of a cathepsin from beef muscle acting optimally at pH 4.1. The purification of an enzyme from striated rabbit muscle having a maximal activity at pH 5.1 has been reported by Snoke and Neurath.² Hess et al.³ have also found such an enzyme with a pH optimum at 3.0 in extracts of a lymphatic tissue.

The importance of intracellular proteases in the hydrolysis of cellular proteins is generally accepted. The role of such enzymes in the formation of polypeptide derivatives from amino acid amides and esters^{4,5,6} is also evident. The studies mentioned led me to undertake a survey of the proteases of the lens with the hope of elucidating their

functions in intracellular protein metabolism in lens, particularly regarding their role in maintaining transparency. The autolysis of lens homogenate was first noted by Clapp⁷ and later extensively investigated by Krause.⁸ Recently Zeller and Devi⁹ and Devi¹⁰ studied the esterase activity of the homogenate and characterized the enzyme which hydrolyses phenylalanine ethyl ester at pH 6.8 as chymotrypsin-like enzyme, using synthetic amino acid esters as substrates.

This paper describes an attempt to find different proteases occurring in the lens, active under acidic and alkaline conditions.

MATERIALS AND METHODS

Bovine lenses were collected and homogenized according to the method previously described.¹⁰ In this study bovine serum albumin (purchased from Nutritional Biochemical Laboratory) and urea denatured hemoglobin (purchased from Armour and Company, Chicago) made up in 0.1M buffers of varying pH were used as substrates and lens homogenate as the source of the enzyme. Acetate was used in making buffers ranging in pH from 3.0 to 5.5; tris for buffers of pH range 6.0 to 7.5; and glycine-glycine for buffers of pH values above 7.5.

* From the Department of Ophthalmology, College of Physicians and Surgeons, Columbia University. Read at the Eastern Section Meeting of the Association for Research in Ophthalmology, Philadelphia, Feb. 18, 1960.

Proteolytic activity determinations were made essentially according to the method of Anson.¹¹ In a typical experiment 1.0 ml. of substrate (6-percent solution in buffer) and 1.0 ml. of the lens homogenate containing 70 mg. of protein/ml. were added to 1.0 ml. of buffer solution, in a 15 ml. centrifuge tube. The mixture was incubated for two hours at room temperature. After incubation, five ml. of five-percent trichloroacetic acid were added to each reaction mixture. The precipitate was centrifuged and the optical density of the supernatant was determined in the Beckman Spectrophotometer (DV model) at 280 m μ against 3.1 percent trichloroacetic acid solution to measure the extent of proteolysis. A blank containing the enzyme but not the substrate was also incubated for the same length of time, following which trichloroacetic acid and the substrate were added. Activity was expressed as the increase in optical density per hour of the trichloroacetic acid filtrate of the reaction mixture, minus the optical density of trichloroacetic acid filtrate of the blank determination. Experimental conditions are shown in Table I.

RESULTS

pH activity curve. Figure 1 shows the pH activity curve which results from the digestion of the urea denatured hemoglobin or bovine serum albumin by bovine lens homogenate over pH range from 3.0 to 10.0. The digestion of the protein substrate occurs maximally at several pH values, the first optimum being at 3.6 to 3.8, the second at 7.0 and the third at 8.8 to 9.0 (fig. 1). Although three pH maxima occur in the pH activity curve, the extent of proteolysis encountered at these pH maxima are not the same. Maximum degradation (fig. 1) occurs at pH 6.8 to 7.0, followed by an intermediate amount of degradation at pH 3.6 to 3.8. The degree of proteolysis at pH 9.0 is very low when compared with the proteolysis which occurs at the other two pH maxima.

TABLE I

EXPERIMENTAL CONDITIONS FOR THE MEASUREMENT OF PROTEASE ACTIVITY OF LENS HOMOGENATE

- | |
|---|
| 1) 1.0 ml. of 6.0% solution of urea-denatured hemoglobin or bovine serum albumin made in 0.1 M buffer |
| 2) 1.0 ml. lens homogenate (70 mg. of protein/ml.) |
| 3) 1.0 ml. buffer |

Time of incubation—2 hours.

Temperature of incubation 28°C \pm 0.5°C.

The occurrence of three pH optima in the pH activity curve might indicate the presence of three separate enzymes in lens homogenate, and certainly there is need of further characterization of these enzyme components.

Proteolytic activity of lens homogenates when prepared in different solvents. Various solvents were tried in the preparation of lens homogenate in an attempt to obtain higher activities of the enzyme with pH optimum at 3.8. Table II illustrates the effect on the activity of the enzyme, using several different solvents in preparing the homogenate. The solvents used for homogenization were 0.25M sucrose, 0.9-percent sodium chloride, 0.1M acetate buffer (pH 3.8) and distilled water. Acetate buffer seemed to be the best medium for homogenization as judged by the degree of proteolysis of the denatured hemoglobin occurring at pH 3.8.

Stability of the pH 3.8 enzyme. In keeping with these findings the homogenate, if prepared in acetate buffer (pH 3.8), retains at least one third of its activity up to six weeks when kept in deep freeze (-15 to -20°C); the activity then slowly disappears in eight to 10 weeks (Table II). In contrast to this finding the homogenates, when prepared in other solvents, retain more than two-thirds of their activity up to four weeks under identical conditions. They all, however, become inactive at a much faster rate than the homogenate made in acetate buffer (Table II).

It has also been noted that the activity of the homogenate prepared from either

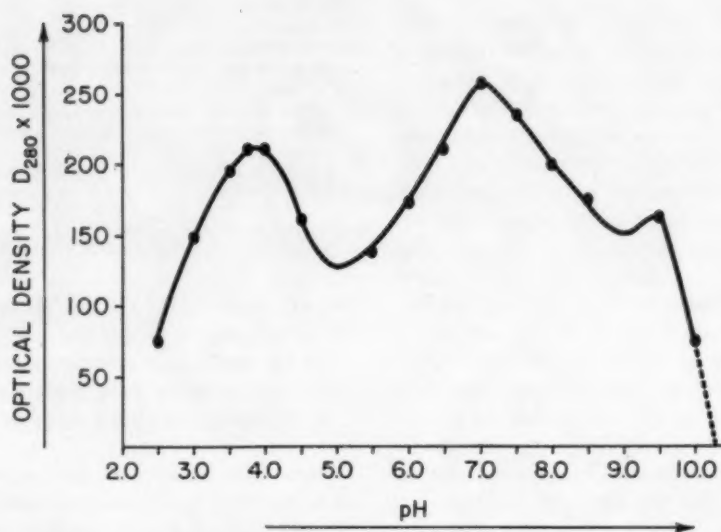


Fig. 1 (Devi). pH-activity curve, showing three maxima, for the digestion of albumin or urea denatured hemoglobin by bovine lens homogenate at 25°C. for two hours.

fresh or frozen lens is practically the same if expressed per mg. of protein N.

Effect of temperature on the rate of reaction. Figure 2 shows a linear increase in the reaction rate, with an increase in the incubation temperature which holds true up to 25°C and then attains a constant velocity. Above 40°C it tends to fall off rapidly.

It also has been noted that maximum proteolysis occurs in two hours of incubation time; this then falls off although some activity was found even after three hours of incubation of the reaction mixture. (fig. 3)

Effect of metallic ions on proteolytic activity of lens homogenate. The activating effect of various metallic ions was tested in the case of the pH 3.8 enzyme. Practically no activation was noted with any of the following metallic ions: Mg^{++} , Mn^{++} , Zn^{++} , or Ca^{++} at $10^{-4}M$ concentration. Cu^{++} and Ni^{++} are both injurious to the enzyme at a concentration above $10^{-3}M$. Prolonged dialysis against 0.005M ethylenediamine tetraacetic acid at pH 8.5 does not indicate the requirement for any metallic ion since inactivation was not observed. Failure to demonstrate at

TABLE II
THE STABILITY OF THE LENS ENZYME (PH 3.8) PREPARED IN DIFFERENT SOLVENTS AT -20°C

Time in Days	$D_{280mg} \times 1,000$			
	Distilled Water (pH 6.0-6.2)	Acetate Buffer (pH 3.8)	Saline (pH 6.8)	Sucrose (pH 6.8)
0 days	192	236	186	193
5 days	125	180	144	140
10 days	75	160	130	125
15 days	25	140	120	94
3 weeks	5 ± 2	120	35	32
6 weeks	—	90	5-10	5-10
8-10 weeks	—	10-15	—	—

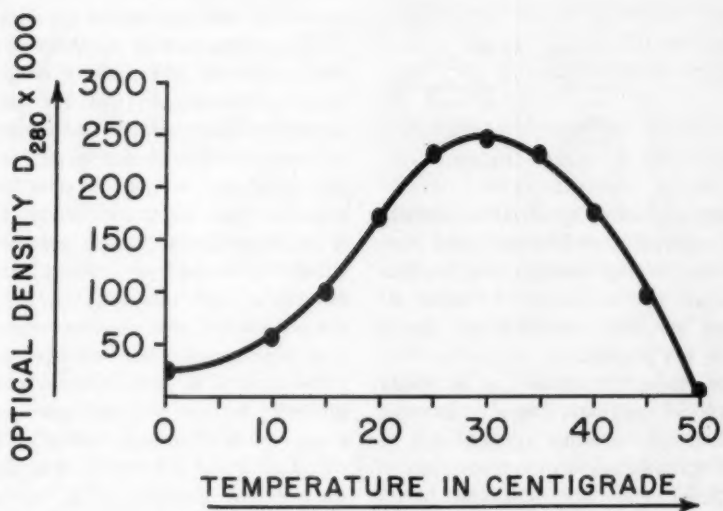


Fig. 2 (Devi). Effect of temperature on the protease activity of bovine lens homogenate at 25°C.

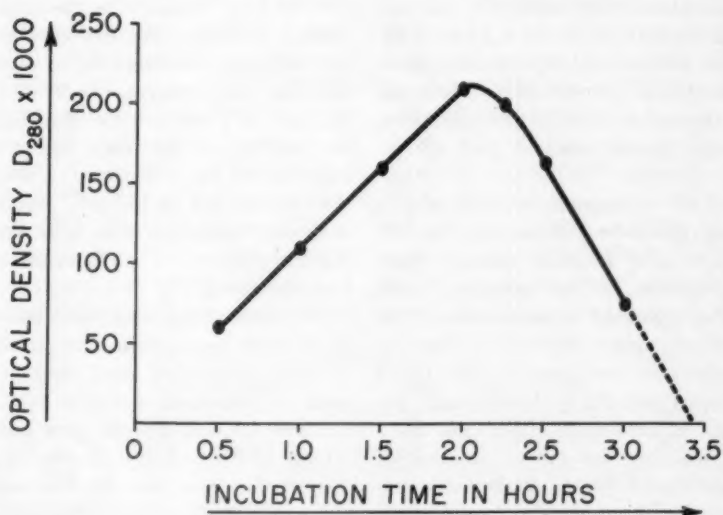


Fig. 3 (Devi). Effect of time of incubation on the protease activity of bovine lens homogenate at 25°C.

TABLE III
AUTOLYSIS OF ENDOGENOUS PROTEINS

Time of incubation	$D_{280m\mu} \times 1,000$	
	Control	Experimental
0 hr.	360	—
1 hr.	498	620
2 hrs.	560	790

least partial activation by divalent metallic ions is not unusual since Morrison and Neurath¹² reported similar findings with erythrocyte proteinase II. The results of Hess et al.³ who studied lymphatic protease are also in accord with my results.

Requirement of -SH group for enzymatic activity (pH 3.8 enzyme). A study was made of the effect of reducing agents, such as cysteine or glutathione, on the hydrolysis of urea denatured hemoglobin, catalyzed by the enzyme in dialyzed lens homogenate acting at pH 3.8. The addition of cysteine or glutathione at a final concentration of 0.01M increases the hydrolytic activity 30 to 35 percent. In complementary studies it was observed that the activity of the enzyme is inhibited 60 to 70 percent by p-chloromercuric benzoate at 0.01M concentration. These results lend support to the idea that sulfhydryl groups might be an integral part of the enzyme.

Autolysis of endogenous proteins in lens homogenate caused by lens enzymes. It will appear from Table III that although there is no degradation of the substrate (urea denatured hemoglobin) in the absence of the enzyme (homogenate), there is an appreciable hydrolysis in two hours at pH 3.8 of the endogenous proteins by the enzyme. That this process is continuous is evident by a significant rise in the optical density of trichloroacetic acid filtrate, containing only the homogenate. Autolysis also occurs at pH 7.0. These results support the original observations of Clapp⁷ and Krause⁸ who noted autolysis of lens homogenate at room temperature. It is also implied from these find-

ings that endogenous proteins present in lens homogenate can be equally good substrates for the enzymes in question.

Characterization of the enzyme. Although the proteolytic pH activity curve showing three pH maxima (fig. 1) provides important evidence of the occurrence of three enzymes in lens homogenate, it was felt that it would be necessary to characterize these enzymes more completely. To do this, it is advantageous to use specific synthetic substrates whose hydrolysis is brought about only by a particular enzyme. For detecting the presence of a pepsin-like enzyme in crude lens homogenate, the homogenate was incubated at 37°C with 0.05 carbobenzoxy-L-glutamyl-L-tyrosine as substrate, in the presence of 0.03M acetate buffer at pH values of 3.0, 5.0 and 6.0. After centrifugation of the reaction mixtures, 25 μ l. of each supernatant was subjected to one dimensional descending paper chromatography. The chromatograms were made using Whatman number one paper, with butanol-acetic acid water (4 : 1 : 5 by volume) in the upper solvent phase. Tyrosine, the expected product of hydrolysis of the substrate in question, was detected by spraying with 0.5 percent (weight by volume) of ninhydrin in 95-percent alcohol. In all cases, except where incubation of the enzyme with the substrate was carried out at pH 6.0, purple spots of different intensities were obtained, indicating the presence of a pepsin-like enzyme in lens homogenate.

To detect chymotrypsin-like enzyme, crude lens homogenate was incubated with N-acetyl-L-tyrosine ethyl ester in the presence of phosphate buffer at pH values of 6.0 and 7.0, and glycylglycine buffer at pH values of 8.0 and 9.0. At centrifugation of the reaction mixtures the four supernatants were separately subjected to paper chromatography using as solvent butanol-ethanol water (4 : 1 : 5 by volume) in the upper phase. The Pauly reagent¹³ was used for spraying the paper to detect N-acetyl-L-

tyrosine as well as the undegraded ester. With the exception of the sample derived from the pH 9.0 incubation, the chromatograms showed orange-colored spots (*R_f* for degraded product 0.2, and for the undegraded material 0.9) of different intensities, indicating the presence of a chymotrypsin-like enzyme in the lens (cathepsin C).

For detection of trypsin-like enzyme in lens homogenate the latter was incubated with benzoyl-L-arginine methyl ester at pH 6.0, 7.0, 8.0, and 9.0. Butanol-pyrimidin water (4:5:1) was employed as solvent, and Sakaguchi reagent¹⁴ was used as a spray reagent to detect benzoyl-L-arginine as well as undegraded ester. No spot corresponding to benzoyl-L-arginine was detected, indicating the absence of a trypsin-like enzyme in lens homogenate.

DISCUSSION

These findings then indicate that lens protein is a relatively rich source of enzymes with proteolytic activity showing three pH maxima. Human gastric juice,^{15, 16, 17} swine,¹⁸ calf gastric¹⁹ mucosal extracts have recently been shown to digest proteins with two and sometimes three pH maxima below pH 5.0. According to the investigators reporting the work, these two proteolytic pH maxima are not the result of two separate enzymes but rather indicate that one and the same enzyme having two active centers is involved. Schlammouritz and Peterson²⁰ also demonstrated two pH maxima below pH 5.0 in the case of crystalline pepsin and that the positions

of the maxima depended upon the nature of the substrates. For example, peptic activity using native substrate (protein) is optimal at pH 1.7 to 2.0, whereas in the case of the denatured protein it is optimal at pH 3.5.

With lens homogenate, however, one obtains three pH maxima which are too widely separated from each other which may more probably correspond to different enzymes each attacking protein substrate optimally at different pH values. The same inference can be drawn from the fact that the lens homogenate can hydrolyze only one type of synthetic substrate at each of the pH maxima (table IV). The enzyme which acts on carbobenzoxy-L-glutamyl-L-tyrosine at pH 3.6 to 3.8 appears similar to pepsin and the second enzyme which hydrolyzes N-acetyl-L-tyrosine ethyl ester at pH 7.0 similar to chymotrypsin.

The autolysis of endogenous lens proteins by its own proteases as measured by an increase in absorption at 280 mμ was based on the procedure developed by Anson¹¹ and subsequently widely used by Dannenberg and Smith,⁴ Snoke and Neurath² in their studies on proteolytic enzymes of lung and striated rabbit muscle and by many others on similar investigation. Recently Koszalka and Miller²¹ excluded the nuclease action in their experiments on the measurement of the proteolytic activity of dystrophic muscle of rat by the same Anson's procedure. In the experiments described in this paper where the autolysis of endogenous proteins

TABLE IV
CHARACTERIZATION OF PROTEASES IN BOVINE LENS

Substrates	Reagent Used to Develop Color of Chromatograms	Colored Spot	Enzyme
Carbobenzoxy-L-glutamyl-L-tyrosine (for pepsin)	Ninhydrin for tyrosin	Gray-bluish	Pepsin-like
N-acetyl-L-tyrosine ethyl ester (for chymotrypsin)	Pauly-reagent-for tyrosine derivatives	Orange brown with different <i>R_f</i> values 0.9, 0.2	Chymotrypsin like
Benzoyl-L-arginine methyl ester (for trypsin)	Sakaguchi-reagent for Arginine derivative	No colored spot	No trypsin like enzyme

was measured, the trichloroacetic acid filtrates, after 2 hours' incubation without the addition of the substrate (denatured hemoglobin), were tested for ribonucleotides and deoxyribonucleotides by using orcinol test for the former and Dische's reagent for the latter. These nucleotides were not found, indicating the absence of any nuclease action under the conditions of the experiment.

The presence of two types of proteolytic enzymes in the lens also was reported by Krause. The β -protease was found by him to be active between pH 7.0 and 4.0; the other α -protease was found to be active between pH 8.0 and 3.0, but the nature of the enzymes was not mentioned. Hanson et al.²² did not find the existence of such an enzyme. However, he did report the presence of an aminopeptidase in the lens, capable of degrading synthetic di- and tripeptides containing glycine as N-terminal residue. His failure to detect any cathepsin-like enzyme due to the use of the supernatant obtained after centrifugation of lens homogenate as the source of the enzyme.

The ability of an enzyme in lens homogenate to hydrolyze phenylalanine ethyl ester at pH 7.0 was reported first by Zeller and Devi⁹ and subsequently was characterized more completely by Devi.¹⁰ This enzyme, which is similar to chymotrypsin in substrate specificity, was designated as cathepsin C. The present studies were undertaken using protein as substrate (urea-denatured hemoglobin) and were made over a pH range of 3.0 to 9.0 to ascertain the total number of proteolytic enzymes that might be present in lens homogenate. All of the enzymes encountered in this study are of intracellular origin and therefore designated as cathepsin-like enzymes.

The importance of intracellular proteolytic enzymes in the development of certain types of cataracts was recognized by Burdon-Cooper,²³ Goldschmidt²⁴ and Labbe and Lavagna.²⁵ Zeller and Devi⁹ recently reported a gradual loss of proteolytic activity

in the lens during the progressive development of cataract. These findings might indicate the importance of proteolytic enzymes in the maintenance of transparency in eye lens; perhaps they accomplish this by degrading the denatured protein as soon as it is formed. These proteases acting under acidic and alkaline conditions play an important role in the intracellular protein metabolism in eye lens.

SUMMARY

1. The occurrence of three different pH optima, widely separated, in the pH-activity curve which resulted from the digestion of the urea-denatured hemoglobin or bovine serum albumin by bovine lens homogenates over a pH range of 3.0 to 10.0 indicated the presence of three different proteolytic enzymes.

2. The optimum temperature of the digestion of urea-denatured hemoglobin by these proteases was found to be in the neighborhood of 28°C and an incubation time of 2 hours was required for maximum proteolysis.

3. Mg, Mn, Zn, or Ca did not stimulate the activity of these proteases, whereas Cu and Ni were both injurious to the enzymes at a concentration above 10⁻³M.

4. Cysteine and glutathione increased the proteolytic activity of these enzymes by 30 to 35 percent when dialysed lens homogenate was used as the source of the enzymes.

5. Appreciable extent of hydrolysis of endogenous lens protein could be carried out by the proteases present in the lens homogenate on standing even at room temperature.

6. The two proteases present in the lens homogenate and acting maximally at pH 3.8 and 7.0 were characterized by studying the hydrolysis of specific synthetic amino-acid esters, and they were found to be very similar to cathepsin A (pH optimum at 3.8) and cathepsin C (pH optimum at 7.0). The third one, which acted at pH 9.0, was not characterized for lack of specific substrates.

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THE INCORPORATION OF LABELLED AMINO ACIDS INTO LENS PROTEIN OF NORMAL, GALACTOSE, AND XYLOSE-FED RATS*

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In the immediate post weanling period, the ocular lens of the rat develops and enlarges at a relatively rapid rate. During this period of enhanced growth, carbohydrate and protein metabolism proceed at an accelerated pace compared with the lens of the older animal.¹⁻⁴ The feeding of a high galactose diet to these young animals will result in the development of dense permanent lenticular opacification in 9 to 14 days.^{1,2,8,9} The effects of a 30 percent xylose diet are quite different. Lens opacities become manifest in 8 to 14 days but these changes are neither progressive nor permanent in spite of the continued ingestion of xylose, and an apparent regression of the cataractous changes eventually occurs.⁶⁻⁸

Some of the biochemical alterations associated with the development of lenticular opacification in the young rat maintained on a galactose or xylose diet have already been elaborated.^{1,2,8,7,8,9,10} In the lens from the galactose fed rat there is an apparent inhibition of the synthesis of soluble lens protein which first becomes manifest about 6 to 8 days after the commencement of the galactose diet. This effect is reflected mainly by the constancy of the soluble protein level in these lenses compared with the rapid increase in this protein fraction in the lens of the normal rat.^{1,9} A similar but less marked effect can be observed in the level of soluble lens protein from the xylose fed animal during the first 14 days on this diet, followed by a slow recovery towards normal values by

the 21st day.⁸ However, the decreased levels of soluble lens protein in these animals (galactose and xylose-fed rats) may simply be due to a lowered food intake during the experimental period rather than a true inhibition of protein synthesis.^{7,11} In order to investigate the effects of these two cataractogenic sugars on protein metabolism in the rat lens, the incorporation of C¹⁴ amino acids into lens protein was studied in lenses derived from normal, galactose and xylose-fed rats, by means of *in vivo* and *in vitro* experiments.

MATERIALS AND METHODS

Holtzman strain white male rats aged 28-33 days and weighing approximately 60 grams each were employed in all the experiments.

The experimental diets consisted of a 60 percent galactose⁵ or 30 percent xylose diet⁸ while the control animals were kept on a glucose diet⁵ and all the animals were maintained on their respective diets for 6½ days.

For the *in vivo* studies 0.2 ml of C¹⁴ leucine (specific activity 7.99×10^5 c.p.m./ μ m), valine (specific activity 7.66×10^5 c.p.m./ μ m) or histidine (specific activity 3.66×10^5 c.p.m./ μ m) was injected intraperitoneally after the animals had been on their diets for 6½ days, and most of the rats were killed three hours later; a small group were kept alive for 24 hours prior to sacrifice. The lenses were removed immediately after the animals were sacrificed, dried by careful blotting with filter paper, weighed, and homogenized. Each pair of lenses was homogenized in 5 ml 5 percent TCA in individual Dounce microhomogenizers¹² and were kept in the cold for 1 hour prior to centrifugation in a refrigerated centrifuge at 3000 r.p.m. for 30 minutes. The precipitate

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was washed twice with 5 percent TCA (5 ml), twice with absolute alcohol (5 ml) and twice with ether (5 ml) and then plated on an aluminum planchet, dried, weighed, and counted in an end window gas flow counter (Nuclear Chicago). Suitable corrections were made for background and self absorption.

For those *in vivo* experiments in which the incorporation of C^{14} amino acids into the soluble and insoluble protein fractions was studied the following procedure was employed. Three hours after the amino acid was injected intraperitoneally the animals were killed and their lenses immediately removed, dried, weighed and homogenized in a solution consisting of 1 part KCl (0.025 M), 1 part $KHCO_3$ (0.035 M), 7 parts sucrose (0.03 M) and 1 part distilled water. Six or 8 lenses were pooled and the volume of solution employed in the homogenizer was adjusted to give a concentration of 50 mgm wet weight of lens per ml of solution. After centrifugation (in a refrigerated centrifuge) at 3000 r.p.m. for 30 minutes the precipitate and supernatant were retained. Fifty percent TCA was added to the supernatant in a volume adjusted to give a final concentration of 5 percent TCA. This material was again centrifuged and the precipitate was washed twice with 5 percent TCA (5 ml), twice with absolute alcohol (5 ml) and twice with ether (5 ml) and then plated, dried and counted. Suitable corrections were made for background and self absorption.

The original precipitate was washed 3 times with 5 ml of the homogenizing media, resuspended, and again homogenized in this media. Five percent TCA was added and after centrifugation the precipitate was washed twice with five percent TCA, twice with absolute alcohol and twice with ether, plated, dried and counted as previously described.

For the *in vitro* experiments the animals were killed 6½ days after they had been on their respective diets, their lenses were immediately removed, dried and weighed. Each

pair of lenses was homogenized in the media described above in a volume adjusted to give a final concentration of 50 mgm wet weight of lens per ml of solution. To 0.5 ml of this homogenate the following reagents were added; 0.15 ml Tris buffer pH 8.5 (0.1 M), 0.05 ml Mg^{++} (0.1 M), 0.1 ml ATP* (5 mgm/ml), 0.1 ml GTP* (2 mgm/ml) and 0.05 ml of either C^{14} leucine, valine, or histidine, and 0.05 ml distilled water. After 1 hour incubation at 37°C in air, 5 percent TCA was added and the precipitate treated in the same manner as previously described for the *in vivo* experiments in which the incorporation of C^{14} amino acids into total lens protein was studied.

In view of the marked activating effect of cobalt on the rat lens amino acid incorporating system (unpublished data) this experiment was repeated with 54 lenses (18 from each of the control, galactose, and xylose-fed rats) in which the 0.5 ml 0.1 M cobaltous chloride (Co^{++}) replaced the Mg^{++} in the reaction mixture, GTP was deleted, and 0.1 ml of distilled water was added. The rest of the experimental procedure remained unchanged.

RESULTS

The results of the experiment in which the degree of incorporation of C^{14} leucine, valine and histidine into lens protein was studied are shown in Table I. In the galactose fed rat the three hour incorporation of C^{14} leucine, valine and histidine into the protein of the rat lens was approximately 30 percent lower as compared with the control animal. In the xylose-fed rat there was a variable effect. While the incorporation of C^{14} leucine and histidine was lower, the incorporation of C^{14} valine was actually higher than in the control animal. However, there is insufficient data for a statistical analysis of the results expressed in Table I and this data can only be interpreted as indicating a

* Both ATP and GTP were obtained from Sigma Chemical Company.

TABLE I

THE IN VIVO INCORPORATION OF C^{14} LEUCINE, VALINE, AND HISTIDINE INTO LENS PROTEIN OF THE NORMAL, GALACTOSE AND XYLOSE-FED RAT; 3 HR. AND 24 HR. STUDY. THE VALUES ARE EXPRESSED AS C.P.M. PER MG. PROTEIN RECOVERED IN EACH PLANCHET

Amino Acid	Control		Galactose		Xylose	
	3 hr.	24 hr.	3 hr.	24 hr.	3 hr.	24 hr.
Leucine	11.6 10.9	4.3	6.0 8.3	4.3	7.1 8.8	3.1
Valine	10.6 8.4	4.4	6.7 6.8	1.5	7.0 13.4	4.0
Histidine	9.3 11.1	7.2	7.2 7.3	2.8	7.1 9.5	8.1

decrease in the incorporation of C^{14} leucine, valine and histidine into the protein of the lens from the galactose-fed animal while the effect of xylose on lens protein is questionable.

The results of the 24 hour studies show a considerable decline in the retained activity with the control, galactose and xylose fed rat lenses. Although only single experiments were done with each amino acid the data indicates that in the lenses from all three groups of rats there is no marked difference in the turnover of the three amino acids employed.

A more significant evaluation can be derived from Table II which shows the results obtained from a similar study in which the lens protein was separated into the soluble and insoluble fractions. In the galactose fed rat, there is a significant decline in the incorporation of all three amino acids into the soluble lens protein fraction ($P < 0.01$ in each) as compared with the results obtained with the control animal. In the xylose-fed rat there is no significant difference in the incorporation of any of the amino acids ($P > 0.05$ in all three cases) as compared with the control lens. With respect to the insoluble lens protein fraction, there was a great deal of individual variation but there was no significant difference in the degree of incorporation of C^{14} leucine, valine or histidine into this fraction from the control, galactose or

xylose-fed rat lens.

The results of the in vitro studies are shown in Tables III and IV. In the experiments in which the reaction mixture contained Mg^{++} rather than cobalt, there was no change in the in vitro incorporation of C^{14} leucine, valine, and histidine into the lens protein of the galactose-fed rats as compared with the control animal (Table III). There was, however, an inhibition of this incorporating system in the lens from the xylose-fed rats. When Co^{++} replaced Mg^{++} in the reaction mixture there was no change in the degree of incorporation of the three amino acids into the lens protein of the galactose and xylose-fed rats as compared with the control group (Table IV). The marked increase in the degree of amino acid incorporation by Co^{++} was evident in all three groups of lenses.

DISCUSSION

The results of the foregoing experiments correlate well with the observations previously reported regarding the biochemical alterations which occur in the lens of the galactose-fed rat.^{1,2,3,9} The significant decline (in vivo) of the incorporation of C^{14} leucine, valine, and histidine, into the lens protein of such animals provides further evidence regarding the inhibition of protein synthesis in these organs. Furthermore these results would tend to refute the possibility that the decreased protein level in the lens from the

TABLE II

THE IN VIVO 3 HR. INCORPORATION OF C^{14} LEUCINE, VALINE AND HISTIDINE INTO THE SOLUBLE LENS PROTEIN FRACTION FROM NORMAL, GALACTOSE AND XYLOSE-FED RATS. THE VALUES ARE EXPRESSED AS C.P.M./MGM. PROTEIN RECOVERED IN EACH PLANCHET

Amino Acid	Control	Galactose	Xylose
Leucine	14.09	7.35	lost
	9.31	8.62	14.90
	11.91	7.67	12.21
	8.93	7.08	11.78
	12.04	8.21	12.88
	9.53	8.28	7.28
	mean = 10.97 S.D. \pm 1.91	mean = 7.87 S.D. \pm 0.189 P < 0.01	mean = 11.81 S.D. \pm 2.46 P > 0.05
Valine	12.57	5.71	12.45
	12.90	6.57	10.19
	9.82	5.00	9.08
	8.36	7.61	9.43
	8.93	6.52	10.14
	10.38	8.15	12.17
	mean = 10.49 S.D. \pm 1.71	mean = 6.59 S.D. \pm 1.08 P < 0.01	mean = 10.56 S.D. \pm 1.29 P > 0.05
Histidine	14.40	6.10	12.48
	13.34	8.79	11.32
	12.04	9.31	10.34
	12.70	6.82	13.13
	12.70	7.44	14.02
	12.60	8.18	11.13
	mean = 12.96 S.D. \pm 0.87	mean = 7.77 S.D. \pm 1.11 P < 0.01	mean = 12.07 S.D. \pm 1.25 P > 0.05

TABLE III

THE IN VITRO 3 HR. INCORPORATION OF C^{14} LEUCINE, VALINE AND HISTIDINE INTO LENS PROTEIN OF THE NORMAL, GALACTOSE AND XYLOSE-FED RAT. THE VALUES ARE EXPRESSED AS C.P.M./MGM. PROTEIN RECOVERED IN EACH PLANCHET

Amino Acid	Control	Galactose	Xylose
Leucine	21.4	26.3	18.7
	33.9	26.0	17.6
	25.2	23.3	20.2
	mean = 26.8	mean = 25.2	mean = 18.8
Valine	16.6	15.2	11.5
	18.6	13.5	12.7
	17.3	15.3	15.5
	mean = 17.5	mean = 14.6	mean = 13.2
Histidine	22.5	20.7	14.2
	22.5	22.8	15.3
	21.6	23.1	15.9
	mean = 22.2	mean = 22.2	mean = 15.1

TABLE IV

THE EFFECT OF Co^{++} ON THE IN VITRO 3 HR. INCORPORATION OF C^{14} LEUCINE, VALINE, AND HISTIDINE INTO LENS PROTEIN OF THE NORMAL, GALACTOSE AND XYLOSE-FED RAT. THE VALUES ARE EXPRESSED AS C.P.M. PER MG. PROTEIN RECOVERED IN EACH PLANCHET

Amino Acid	Control	Galactose	Xylose
Leucine	207.7	228.9	158.2
	199.3	184.7	191.6
	195.4	166.4	164.1
	mean = 200.8	mean = 193.3	mean = 171.9
Valine	142.0	196.3	140.0
	138.1	186.8	153.6
	148.2	210.1	165.3
	mean = 142.8	mean = 197.6	mean = 154.3
Histidine	595.5	770.0	635.1
	590.6	680.6	594.5
	672.6	652.7	749.2
	mean = 622.9	mean = 701.1	mean = 659.6

galactose-fed rat is simply due to a lowered food intake during the experimental period.

The effect of a 30 percent xylose diet on the level of soluble lens protein is much less marked than that of galactose and the cataractogenic action of this sugar is also less severe and more variable.⁸ This effect is further reflected by the results of the present experiments. It would thus appear that the cataractogenic action of xylose is more complex than that of galactose. The biochemical alterations occurring in the lens of the xylose-fed rat are further complicated by the fact that this organ is capable of adapting towards the deleterious effects of this sugar with regression of the lenticular changes occurring after the rat has been on the diet for 2-3 week.⁶⁻⁸

The results of the in vitro experiments with the lenses derived from the galactose-fed rats indicates that there is no basic impairment in the amino acid-protein incorporating system in this organ when sufficient exogenous ATP is added. This finding is in agreement with the earlier observation that there is a marked decline in ATP in such lenses (3). Apparently these lenses (at least after 6 days on the galactose diet) can incorporate amino acids at a normal rate if sufficient ATP is present. The situation with respect to the

lenses from the xylose-fed animal is somewhat more complex. In the in vitro system where Mg^{++} was employed, there was an apparent inhibition of amino acid incorporation. When, however, Co^{++} was employed the incorporation proceeded at a relatively normal rate. From experiments recently completed in our laboratory it would appear that the activating effect of Co^{++} is a true one rather than an artefact. Furthermore its major action appears to be directed at the RNA-amino acid incorporating system of the rat lens although it also causes a definite increase in amino acid incorporation into lens protein (unpublished data). The activating effect of cobalt on the RNA-amino acid incorporating system thus may explain the apparent reversal of the inhibition of this system observed in the in vitro experiments in which Mg^{++} was employed.

Previous experiments have indicated that the level of RNA in the lens of the galactose-fed rat shows no change as compared with the normal rat lens.³ A similar situation exists with respect to the RNA content of the xylose-fed rat lens (unpublished data). However the level of soluble lens protein in such animals shows a relative decrease as compared with the normal^{1,6} and this effect is most marked in the lens of the

galactose-fed rat. This indication of an apparent inhibition of soluble protein synthesis in the lens of the galactose fed animal has been further strengthened by the results of the present experiments. A further effect in such lenses is the marked fall in ATP concentration which occurs in the galactose-fed rat lens and a moderate temporary decline which occurs in the lens of the xylose-fed animal.^{3,6} It is thus not surprising that protein synthesis might be markedly affected in the former situation as is borne out by the results of the *in vivo* experiments.

SUMMARY

There is a significant inhibition in the *in vivo* incorporation of C¹⁴ leucine, valine, and histidine, into the protein-RNA complex and soluble protein fraction of the lenses from rats maintained on a cataractogenic galactose diet for 6½ days.

The *in vitro* incorporation of these three amino acids into the protein-RNA complex of such lenses is not impaired.

The effect of a xylose-diet on the incorporation of C¹⁴ leucine, valine, and histidine, into the lens protein-RNA complex is much more variable.

There is no significant difference between the incorporation of these three amino acids into the soluble protein fraction of the lens from the xylose-fed animal as compared with the normal.

Cobalt has a marked activating effect on the *in vitro* incorporation of C¹⁴ leucine, valine, and histidine into lens protein.

These results are considered and compared with the biochemical alterations which previously have been reported to occur in the lenses from galactose and xylose-fed rats.

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OCULAR UPTAKE OF RADIOTHYROXINE AND RADIOTRIIODOTHYRONINE

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Recent evidence indicates that the hypometabolism observed in certain patients with uveitis may be related to inefficient utilization of thyroid hormones by peripheral tissues.¹ In attempting to relate this finding to levels of metabolism within the eye, it is necessary to study the ocular uptake of thyroid hormones as a preliminary step.

For this purpose, an attempt has been made to study the distribution of radiothyroxine and radiotriiodothyronine in the anterior segment of the rabbit eye, with and without induced uveitis.

METHODS

Young albino rabbits, 1600 to 2000 grams, were injected with 50 microcuries of radiothyroxine and radiotriiodothyronine. The radio-chemical purity was checked by paper chromatography prior to, and following, shipment. The total ocular uptake of the labeled thyroid hormones was expressed as the Differential Absorption Ratio. (fig. 1) The geometric efficiency of the well-scintillation counter system used in this study was 0.25.

In studying the ocular uptake of these hormones, four control eyes and four eyes with uveitis were measured at various times between one and 96 hours.

Ocular tissue samples were recovered under Nembutal anesthesia. Aqueous samples were delivered by paracentesis, the iris evulsed through a limbic incision and the lens removed intracapsularly. The cornea was excised completely.

Each tissue sample was weighed and pre-

pared by the wet-ash method (digestion in fuming nitric acid). Counting samples were placed in glass tubes and radioactivity was measured in a well-type scintillation counter.

Uveal inflammation was induced anaphylactically in selected rabbits by subcutaneous injection of sterile horse serum, emulsified with Freund's adjuvant. The sensitizing dose injected was two milliliters. Shock doses of 0.1 ml. of sterile horse serum were injected into the vitreous after seven to ten days.

Half-time turnover studies of triiodothyronine and thyroxine were also carried out. Six rabbits were injected with 50 microcuries of each hormone in 50 percent propylene glycol solution. Three samples of 1:1,000 dilution of the injected dose were also made up as standard counting samples. The volume of these was 1.0 ml. At appropriate intervals during 14 days, blood samples were recovered by cardiac puncture. The plasma samples were derived following centrifugation. Plasma samples were labeled and stored in the refrigerator until day 14. At this time, all samples and standard preparations were counted. The disappearance rate of radioactivity from the peripheral blood was plotted semi-logarithmically and the half time of turnover for each hormone was derived from intersecting co-ordinates drawn from this curve as shown in Figure 2.

RESULTS

The two hormones are known to have vastly different rates of diffusion throughout the body.² As shown in Figure 2, the triiodothyronine moves rapidly from the blood stream during the first hour after injection so that beyond this point, blood levels of thyroxine available for tissue uptake are consistently higher than in the case with the other hormone. This diagram shows that the triiodothyronine half time is slightly more

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than two days in this series while the thyroxine half time is closer to four days.

This difference in turnover rates probably also affects the magnitude of total ocular uptake. The ocular DAR (Differential Absorption Ratio) of radiothyroxine at one hour interval is three times that of the radiotriiodothyronine value (fig. 3). The difference at the 72 hour interval is even greater: thyroxine here exceeds triiodothyronine by a factor of 15.

Having thus noted the primary differences in turnover rate and ocular uptake between the two hormones, it was next attempted to examine the ocular distribution of each hormone within the tissues of the anterior segment.

$$\text{DAR} = \frac{\text{microcuries/gram ocular tissue}}{\text{microcuries/gram body weight}}$$

* DIFFERENTIAL ABSORPTION RATIO

Fig. 1 (Pyeatte). Derivation of value for Differential Absorption Ratio (DAR) used to express relative uptake of labeled hormones by various tissues (after Marinelli and Kenney⁸).

Regarding the cornea, highest levels of thyroxine occurred at eight hours after injection in normal eyes, and about 16 hours after injection, with uveitis. The general magnitude of corneal uptake for both thyroxine and triiodothyronine was low in comparison

HORMONE DISAPPEARANCE CURVE per ml. of PLASMA

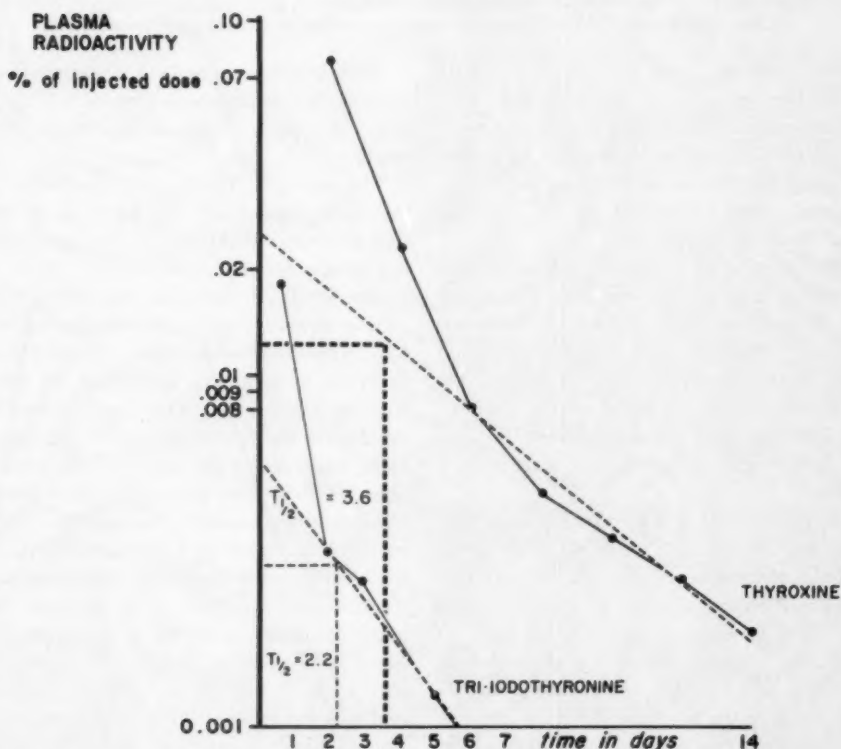


Fig. 2 (Pyeatte). Half-Time turnover of thyroxine and triiodothyronine compared by measurement of changes in plasma levels. Extrapolation of slope to zero time indicated by straight line.

DAR OCULAR TISSUE... each interval represents mean of four rabbit eyes

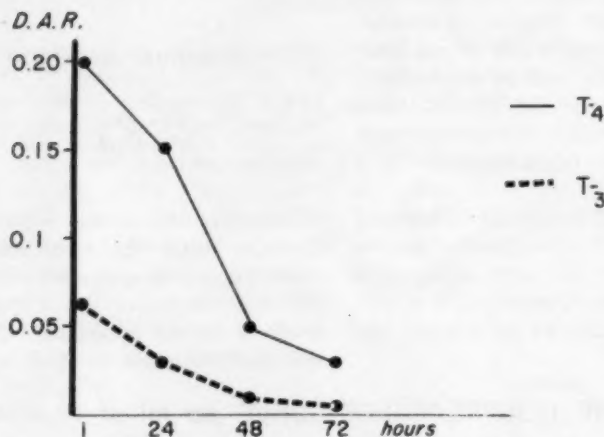


Fig. 3 (Pyeatte). Intact eye levels of thyroxine and triiodothyronine compared according to Differential Absorption Ratio (DAR). Thyroxine levels are greater at all time intervals tested.

to other tissues of the eye, e.g. below 0.01 microcuries per excised whole cornea. This does not imply that uveitis was without effect on corneal uptake, however. As shown in Figure 4a, thyroxine levels in the cornea increased about threefold at all intervals when active uveitis existed.

The retention of triiodothyronine by normal cornea was slightly less than that found with thyroxine. This difference, however, does not reflect the full disparity in blood levels of the two hormones. In uveitis, the blood level pattern is more nearly approximated, that is, with triiodothyronine, the corneal levels with inflammation were nearly doubled.

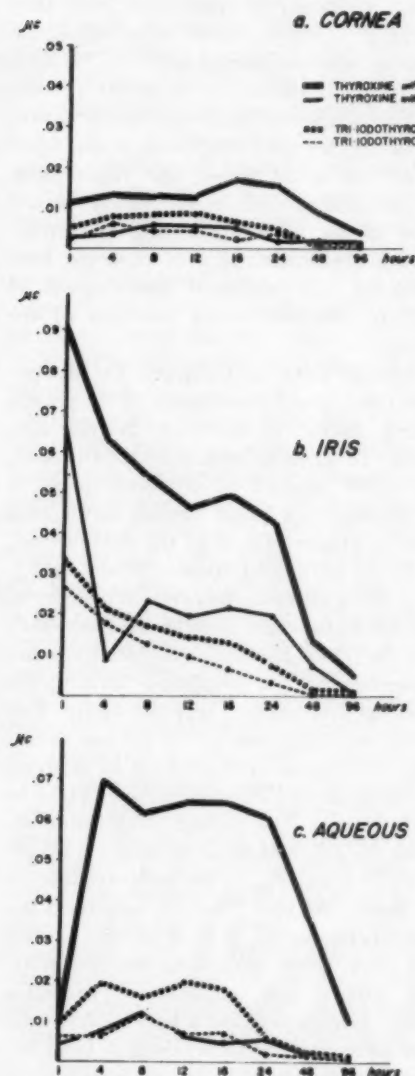
The patterns of iris uptake for each hormone are shown in Figure 4b. Not unexpectedly, thyroxine in the iris exceeds that found in the cornea under all conditions and also follows more closely the pattern of blood level changes. There is a sharp decline at four hours, possibly representing equilibrium phase in the hormone diffusion process, and following this point, a more gradual decline between 24 and 96 hours.

With uveitis, there is a disproportionate increase in iris thyroxine (fig. 4b). This follows the pattern of blood levels rather closely.

The amount of triiodothyronine in the iris under all conditions and at most intervals was considerably lower than corresponding values for thyroxine.

The levels of thyroxine and triiodothyronine in aqueous humor (fig. 4c) were studied for comparison with values obtained previously for cornea, iris, and blood. In normal aqueous humor, thyroxine reaches peak levels during the first eight hours: during this time, blood levels are seen to fall, while the aqueous level rises gradually. With induced inflammation, however, the aqueous thyroxine level is higher and appears to peak earlier. Further, the decline of thyroxine under these conditions does not commence until after 16 hours, showing a departure from blood level changes. Beyond 24 hours, there was a sharp decline in aqueous thyroxine, and to some extent, this accompanied resolution of the inflammation, generally between the second and third day. It also represents

Changes in Ocular Levels of ^{131}I thyroxine and ^{131}I triiodothyronine Compared with and without UVEITIS....



the rapid falling away of blood levels of thyroxine. There was no attempt made to reactivate the uveitis in these animals. During the first 24 hours in the presence of inflammation, the aqueous levels of protein bound

Fig. 4 (Pyeatte). Changes in hormone level within the anterior segment of the eye.

a. Corneal levels of each hormone are lower at all times tested, and show little change in uveitis. Thyroxine exceeds triiodothyronine at all intervals, and shows departure from falling blood levels.

b. Iris levels of each hormone are greater than the cornea, and are in closer agreement with blood levels. Thyroxine exceeds triiodothyronine generally, and shows disproportionately increased retention during induced uveitis.

c. Aqueous levels of each hormone are greater than cornea, and show less correlation with blood levels than iris. Both hormone levels are increased during uveal inflammation, with thyroxine being the higher, and longer in effect.

hormone will rise by a factor of eight or nine when the blood-aqueous barrier is breached by uveitis. The aqueous-blood ratio of thyroxine in normal rabbit eyes ranged between 1:140 and 1:154. In uveitis, it ranged between 1:55 and 1:95.

The situation of triiodothyronine in the aqueous is considerably different. Contrary to what was noted in the iris, in the aqueous there was no major change effected by uveal inflammation, beyond the first hour. The levels under all conditions and at all time intervals were considerably lower in the aqueous than was the case with thyroxine.

Radio-chromatography demonstrates the presence of the radioactive iodine in the same position as is the thyroid hormone, thus establishing that the radioactive element is attached to the hormone after its passage across blood-aqueous barrier (fig. 5).

Initial attempts to study hormone distribution by tracer methods in vitreous and retina were defeated by frequent contamination with adjacent blood. These studies have had to be deferred until better techniques can be devised.

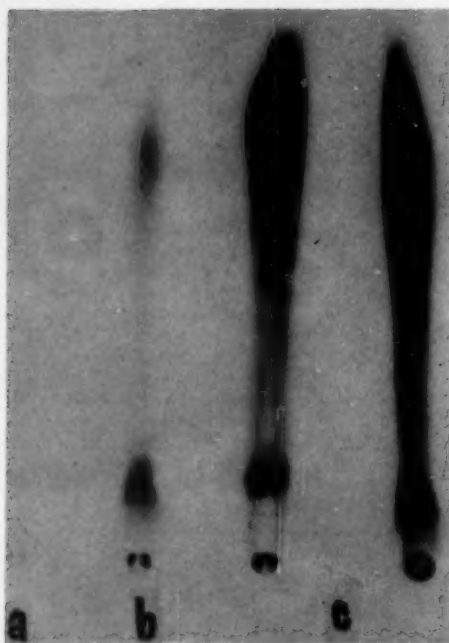


Fig. 5 (Pyeatte). Radiochromatograph of radiothyroxine in Aqueous Humor. a. Standard; b. Primary aqueous; c. Plasmoid (secondary) aqueous. Demonstrates intact protein-bound hormone in primary and secondary aqueous.

DISCUSSION

Tissues of the eye vary widely in their levels of metabolic intensity: the cornea and vitreous being relatively inert in relation to the retina, which, in most species, shows intense metabolism. The place of the uveal tissues in this respect is somewhat uncertain and there is little known about their relative level of metabolism or the relationship that this function might play to resisting or acquiring inflammatory disease. In this respect, the ocular uptake of circulating thyroid hormone, as reported in this paper, represents an initial attempt to study tissue metabolism in relation to uveitis. Overall, the initial impression is that both of the hormones examined (thyroxine and triiodothyronine) exhibit a very rapid turnover in the rabbits. For this reason, there was not a prolonged substantial blood level of either

hormone available for uptake by the ocular tissues beyond the first few days following injection of exogenous hormone. The human system is known to turn over these substances at a much slower rate: thyroxine turnover half-time normal is $5\frac{1}{2}$ to $7\frac{1}{2}$ days and triiodothyronine is about 50 percent faster.³ As reported by Gross,⁴ about 90 percent of injected triiodothyronine is destroyed or excreted in 24 hours. The results with this hormone found in our study accord rather closely with this finding in that insignificant radioactivity was observed both in normal and inflamed eyes beyond 24 hours of triiodothyronine injection of the animal.

Previous work of Crispwell has demonstrated that broad significance of the protein binding factor in studying thyroid hormones.⁶ In terms of general body utilization, the higher potency of triiodothyronine is felt to relate to a looser binding factor, thus allowing greater release of the active principle to the peripheral tissues. When considering ocular uptake, however, triiodothyronine bound to serum globulin does not easily pass the blood-aqueous and blood-vitreous barriers before its circulating level in the blood has dropped to a very low figure. This is one basic explanation for the low differential absorption ratio obtained in studying the thyroxine and triiodothyronine uptake by ocular tissues. The highest DAR value obtained for either of these hormones was only 0.199, or about $\frac{2}{10}$ of the body average (at one hour). At later times, it was much less than this figure. It will be of interest to compare these figures with those obtained in human studies now underway. Triiodothyronine, as expected, has a lower DAR value than thyroxine. Its values ranged from less than $\frac{1}{4}$ of thyroxine at the one hour interval (0.0626), to about $\frac{1}{50}$ at the 72 hour period.

The generally low uptake of the cornea for both hormones is not surprising in view of its avascularity and the low metabolic rate of this tissue. The failure of corneal tissues to parallel a rising level in the aque-

ous humor is unexplained. It is possible that corneal tissues do not easily take up or draw upon supplies of thyroid hormone in adjacent fluid. A moderate rise of radiothyroxine occurred in the face of falling blood levels: this exceeded the level reached with triiodothyronine under similar conditions. There is, thus, some indication that thyroid hormones are retained by the corneal tissues. It is not possible within the limits of this study to say that radioactivity actually represents retention of intact hormones. The studies of von Sallmann have previously shown preferential uptake of free iodine by corneal tissues.⁶ It is possible that the activity shown here represents corneal uptake of iodine liberated by degradation of the hormones after injection.

The markedly increased uptake of thyroxine by the iris tissue, with and without inflammation, represents both a higher tissue metabolism above the corneal level, and, of course, a better blood supply. The iris followed changes in blood levels of radioactivity more closely than did the cornea. Triiodothyronine was noted to fall rapidly in the iris, also reflecting rapid changes in blood level beyond the 24 hour interval.

The aqueous, unlike the iris, shows an eight hour delay in reaching peak levels of thyroxine concentration. This would seem to indicate a time factor in penetrating the blood-aqueous barrier. Also, unlike the iris, a disproportionate and early rise in activity is encountered in the aqueous when uveitis is induced, probably reflecting a breach of the blood-aqueous barrier.

Levels of triiodothyronine, in the aqueous, are noted to be largely unchanged by inflammation, apparently explained on the basis of rapidly falling blood levels of this hormone, as previously discussed.

It thus appears that the levels of thyroxine in the aqueous and iris are greatly increased by inflammation, while the uptake of the cornea is not. These increases reflect largely changes in blood level and breaching of the

blood-aqueous barrier. As shown also, the triiodothyronine content of all tissues is low, indicating, perhaps, that the blood level of circulating hormone is the crucial factor in determining ocular uptake.

There is considerable need to study more about the effects of thyroid hormone on ocular tissues and on the role that this might play in enabling them to resist various inflammations and infections. In other fields, considerable work has already been done on the effects of thyroid hormone in this respect. Perhaps significant in this respect is the work of Lurie which showed that rabbits susceptible to tuberculosis acquired some degree of protection when premedicated with thyroid hormone.⁷ There were no studies of ocular effects reported in his publication.

In relation to the present work, it has recently been shown by Bolla,⁸ to a limited extent, that thyroxine does penetrate into the ocular tissues in approximately the degree shown in this report.

SUMMARY

Total ocular uptake of radiothyroxine and radiotriiodothyronine was found to be low, in comparison with body average DAR, probably due to rapidly decreasing levels of circulating hormone. This is evidenced by a turnover half-time ($T_{1/2}$) of two days for triiodothyronine, and almost four days for thyroxine.

Levels of the hormones in the cornea were lower, both in normal eyes, and in those with uveitis, than seen in the iris and aqueous, reflecting low corneal metabolism and relative avascularity of this structure.

Thyroxine levels were uniformly higher than those of triiodothyronine, in the iris and aqueous, in both normal eyes, and in those with induced uveitis. This is probably due to higher levels of the more tightly protein bound thyroxine, vasodilatation of the iris in inflammation, and breaching of blood-aqueous barrier in uveitis.

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OCULAR DISTENSIBILITY AS A FUNCTION OF TEMPERATURE

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Recent experimental investigations concerning the influence of hypothermia on aqueous humor formation,¹ in which tonometric and tonographic methods were utilized, have indicated the desirability of more precise information regarding the influence of temperature on ocular rigidity.

The present investigation was made for this purpose. In addition, the results have an important bearing on the problem of variations in ocular rigidity in the enucleated globe when compared with measurements made in vivo.

MATERIAL AND METHODS

Adult cats of either sex, ranging from 1.5 to 3 kg., were used for all experiments. A total of 32 enucleated eyes was used in the study.

The experimental protocol was identical

to that reported in a prior publication,² with the following alterations.

1. Since the coefficient, $E = dP/dV$, varies continuously with the initial pressure, a single, constant initial pressure of 25 mm. Hg was selected for the measurement of E in all experiments. As reported previously, E represents the slope of the pressure-volume increment curve at any constant initial pressure, and is equivalent to the spring coefficient of a Hooke-body analogue.

2. In eleven eyes, the temperature of the constant temperature bath was maintained at 37°C, and E measured initially, and at 20, 50, and 95 minutes after commencing the experiment. Usually only approximately 5 minutes elapsed between enucleation and the beginning of the experiment. Similarly, in ten eyes, the temperature was maintained at 7°C and E measured at the same time intervals. In these experiments approximately 10 minutes were allowed for the eye to come into temperature equilibrium. These experiments served as controls for the variation in E as a function of time.

3. In eleven eyes, E was measured at 7°,

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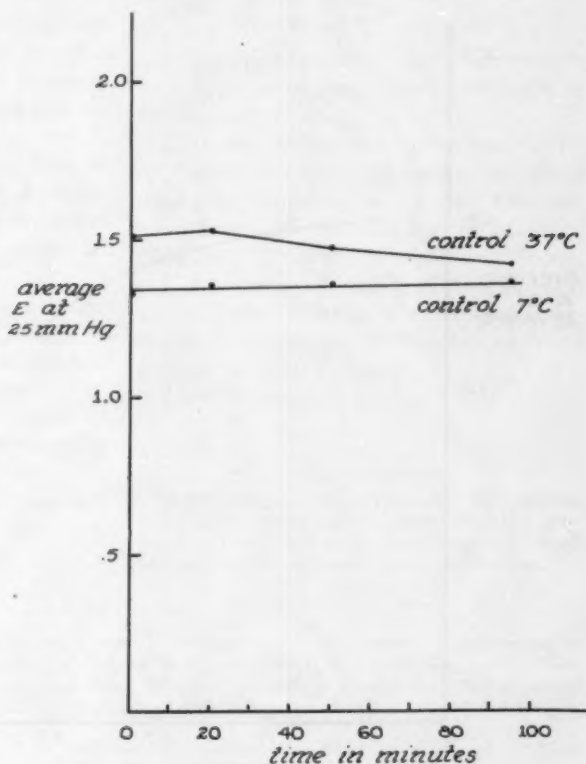


Fig. 1 (Holland, Tischler, and Bellestri). E as a function of time. There is a slight diminution in rigidity following enucleation when the eye is maintained at 37°C , and no significant change at 7°C .

17° , 27° , and 37°C , in this order, maintaining the same time intervals between measurements as in the control experiments. In several experiments the temperature sequence was reversed, but the rate of outflow increased to such a degree over the experimental period that accurate measurement of the pressure increments became difficult. Because of this observation, all experiments in this series began with 7°C .

RESULTS

Figure 1 illustrates the variation in average E as a function of time. When the temperature is maintained at 7°C there is almost no variation in E for 95 minutes, whereas at 37° there is a slight diminution in E of approximately 6 percent. The average E for 37° is higher than that at 7°C in Figure 1 because of random variation in

E for different control groups of eyes.

In Figure 2, average E is represented as a function of temperature. As the eye is cooled from 37° to 27°C the average E increases approximately 11 percent. In cooling the globe from 37° to 7°C the average E increases approximately 18 percent.

A statistical evaluation of the significance of these changes is summarized in Figure 3, where the "t" test is utilized as the measure. The change in slope of the pressure-volume increment curve, ΔE , which occurs on changing the temperature from 7° to 27°C is significant at the level of $P < .001$; P is less than .01 for the change from 27° to 37°C .

DISCUSSION

The results of this investigation indicate that cooling of the eye results in a statistically significant change in ocular distensi-

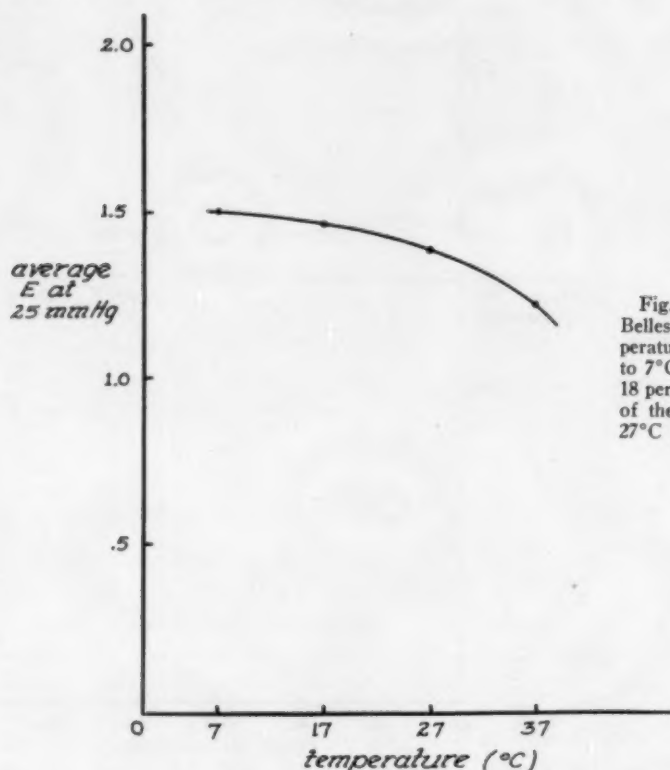


Fig. 2 (Holland, Tischler, and Bellestri). *E* as a function of temperature. Cooling the eye from 37° to 7°C results in approximately an 18 percent increase in rigidity. Most of the change occurs from 37° to 27°C (approximately 11 percent).

bility, and in the expected direction. A -20°C change in temperature results in approximately a 16 percent increase in rigidity. The change is non-linear, with most of it occurring with the first ten degree change. This observation would imply that the effect of hypothermia on aqueous humor formation may be greater than previously estimated.¹

Several reported investigations dealing with the problem of changes in ocular rigidity following enucleation or death are not in agreement. Macri³ Clark,⁴ and Grant,⁵ reported no significant change in rigidity immediately after death or enucleation. Grant, however, also observed a diminution of ocular rigidity during the first hour or two post mortem. Perkins and Gloster⁶ found, in

TEMPERATURE °C	AVERAGE E	t TEST FOR SIGNIFICANCE
7°	1.496	$\Delta E (7^{\circ} \text{ to } 37^{\circ}) P < .001$
17°	1.465	
27°	1.378	$\Delta E (27^{\circ} \text{ to } 37^{\circ}) .001 < P < .01$
37°	1.226	

Fig. 3 (Holland, Tischler, and Bellestri). Statistical tabulation of the significance of the changes in *E* as a function of temperature using the "t" test as a measure.

half of their experiments, that the rigidity was 20 percent higher in the dead eye than in the living. Wessely⁷ reported increased scleral rigidity in the human eye two to six hours post mortem and concluded that it was of the same order of magnitude as in vivo measurements with the Schiotz tonometer. In these experiments on the enucleated globe, the temperature was maintained at 37°C.

From the results of the present investigation it appears probable that changes in ocular rigidity following death or enucleation are compounded of at least two different influences, viz. a slight diminution of rigidity as a result of time (when the globe is maintained at 37°C) and a somewhat greater change in the opposite direction as a result of cooling of the eye from body temperature to room temperature.

SUMMARY AND CONCLUSIONS

1. An investigation of the ocular rigidity as a function of temperature was made on the enucleated cat eye.

2. If the temperature is maintained at 37°C there is a slight diminution in rigidity during the first hour and a half following enucleation. If the temperature is maintained at 7°C there is no significant change.

3. As the temperature of the globe is lowered, the ocular rigidity increases significantly, amounting to approximately 18 percent for a 30°C change.

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SHORT-CIRCUIT CURRENT AND RESISTANCE OF THE IN VITRO IRIS-CILIARY BODY MEMBRANE PREPARATION

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INTRODUCTION

In vitro methods of investigation of biologic systems have proved to be valuable for the understanding of active transport mech-

anisms. The work of H. Ussing with the isolated frog skin is noteworthy in this regard, for he was able to show that the short-

* From the Department of Ophthalmology, Tulane University School of Medicine. This investigation was supported by research grant B-2212, from the

National Institutes of Health, Public Health Service. Read before the Mid-Winter National Meeting of the Association for Research in Ophthalmology, New Orleans, December 5, 1960.

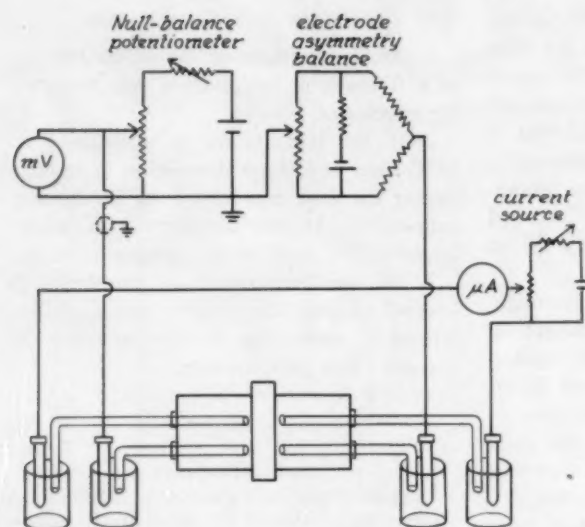


Fig. 1 (Holland, Mallerich, Tischler and Bellestri). A schematic diagram of the measuring circuit.

circuit current generated by the preparation was the result of, and equivalent to, the active transport of sodium ion.¹

The present investigation is one of a planned series directed toward the elucidation and systematic characterization of possible transport systems in the iris-ciliary body membrane preparation. This report is concerned with the short-circuit current, resistance, and power of the cat iris-ciliary body membrane preparation.

MATERIALS AND METHODS

Adult cats of either sex, ranging from 1.5 to 3 kg, were used in all experiments.

The method of preparing the iris-ciliary body of the cat eye as a membrane separating two reservoirs containing identical fluids has been given in a previous publication.² The arrangement of the calomel reference electrodes and measuring system (fig. 1) was similar to that reported by Ussing.¹ Kintel model 203 microvoltmeter was used in the null-balance potentiometric circuit, and Kintel model 204 microammeter in the current measuring circuit.

After balancing electrode asymmetry, the resistance of the Ringer's solution between the voltage KCl-agar bridges was deter-

mined by passing small currents (1-10 μ A) through the solution and measuring the potential between these bridges. This resistance, R_o , was subsequently subtracted from the resistance measured with the membrane in the chamber to give the true membrane resistance. R_o was almost always less than 10 ohms.

The membrane was placed in position and the chambers filled with oxygenated Ringer's solution containing 100 mg percent glucose. The potentiometer was adjusted until an equal and opposite potential was applied, i.e. until null-balance was achieved. This latter potential was recorded on a potentiometric recorder. The impedance of the voltage measuring system was thus very high, thereby drawing a negligible current from the membrane during the measurement. Following the initial potential measurement, a series of small currents (1-10 μ A) was passed through the chamber and the potential changes across the membrane measured. The series of applied currents with their corresponding potential changes (fig. 2) made it possible to calculate the membrane resistance. When the applied current was of the same direction as the current flow in the membrane, it was designated as a for-

ward current—if opposite, a reverse current. It was observed that the resistance thus determined was independent of the direction of the applied current. Thus for simplicity, all resistance measurements reported here were obtained from applying reverse currents.

In all experiments the membrane potential was measured continuously and resistance measured every four minutes. Altogether 30 experiments were made, but in the first eight the initial chamber resistance was not measured. The values reported here are based on the remaining 22 experiments. The time which elapsed from enucleation to measurement of the initial potential was recorded as well as the weight of the membrane preparation at the conclusion of the experiment. The wet weight was determined after removal of excess fluid by gentle blotting. All experiments in this and the previous study² were made at room temperature.

RESULTS

The results are summarized in Figure 3 where the means, standard deviations, and ranges of the resistance, initial potential,

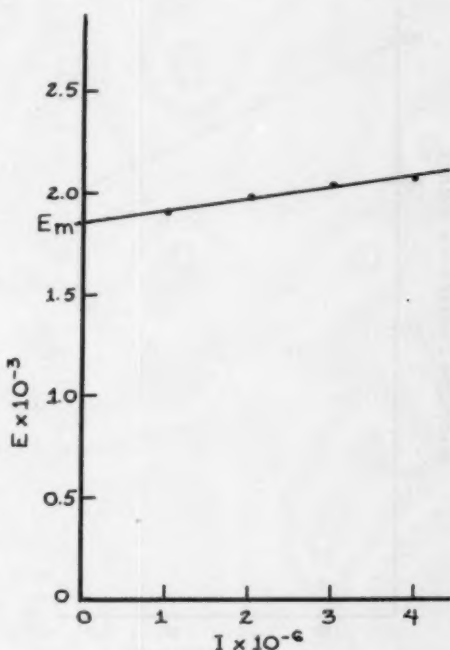


Fig. 2 (Holland, Mallerich, Tischler and Belles-tri). A rectilinear plot of potential versus applied current. The slope of the resulting straight line is the membrane resistance, and the ordinate intercept, the initial membrane potential, E_m .

	RANGE		AVERAGE		STANDARD DEVIATION
	<i>unextra- polated</i>	<i>extra- polated</i>	<i>unextra- polated</i>	<i>extra- polated</i>	
INITIAL POTENTIAL (millivolts)	0.80-2.30	2.59-7.03	1.22	4.62	± 0.45
SHORT CIRCUIT CURRENT (microamperes)	15.5-54.1		29.7		± 10.5
RESISTANCE (ohms)	34.7-51.0		40.7	47.0	± 3.7
POWER (nanowatts)	9.3- 124.5	143.3 - 1051.0	40.8	496.1	± 28.8
WEIGHT (milligram)	199-303		249		± 24

Fig. 3 (Holland, Mallerich, Tischler and Bellestri). Tabular summary of the measured and calculated electrical membrane parameters.

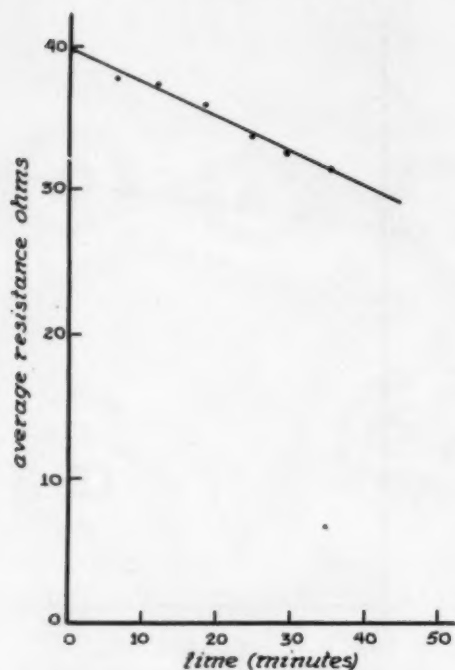


Fig. 4 (Holland, Mallerich, Tischler and Bellestri). Average membrane resistance appears to diminish as a linear function of time.

initial short-circuit current, initial power, and weights are recorded. In the following, all means are given with \pm a standard deviation.

Mean initial membrane resistance was 40.7 ± 3.7 ohms, with a range of 34.7 to 51.0 ohms.

Average initial membrane potential was $+1.2 \pm .45$ millivolts, with a range of $+.60$ to $+2.3$ millivolts, the stromal surface being always positive with respect to the neuroepithelial surface.

The initial short-circuit current averaged 29.7 ± 10.5 μ A, with a range of 15.5 to 54.1 μ A.

Average initial power (E^2/R) was 40.8 nanowatts* ± 28.8 with a range of 9.3 to 124.5 nanowatts.

* A nanowatt is 10^{-9} watts. "Nano" is the prefix for 10^{-9} recently recommended by the National Bureau of Standards.

Mean membrane weight was 249 ± 24 milligrams, with a range of 199 to 303 milligrams.

The average membrane resistance falls slightly during the experiments, and appears to be a linear function of time (fig. 4). If it is assumed that the resistance changes in this manner from the moment of enucleation to the initial measurement, it is possible to estimate the average membrane resistance at the time of enucleation.

By extrapolation, the average membrane resistance is 47 ohms, as recorded in Figure 2.

The membrane power diminishes rapidly during the experiments, and in most experiments appeared to approximate an exponential decay (fig. 5). Using the same assumptions as above, the average power at the moment of enucleation can be approximated roughly by extrapolation. Using the average extrapolated membrane resistance and power, the corresponding membrane potential can be obtained. These values are recorded in Figure 2.

DISCUSSION

In many biologic membranes, the presence of a membrane current has been associated with the movement of ions across the membrane. H. Ussing has been able to show that, in the frog skin, the membrane current is due to the active transport of sodium ion.¹ The measurement of a current in the iris-ciliary body membrane preparation does not mean, necessarily, that it is generated by the active transport of any particular ion species in solution. This might be ascertained by the simultaneous measurement of membrane current and ionic flux as was done by Ussing. Such studies are now in progress using the iris-ciliary body preparation. For the moment, it is of sufficient interest to inquire about the energy requirements for the calculated electrical work and compare this with an estimate of the energy required for aqueous formation based on different methods.

The experiments of Davson, Duke-Elder and Maurice³ are relevant and important, because they show beyond reasonable doubt, that sodium, and very probably chloride, ion concentrations in the cat aqueous humor are not the result of a dialysis equilibrium. The excess of sodium and chloride above a Gibbs-Donnan equilibrium implies the expenditure of energy. The required energy can be estimated from the free energy change necessary for the difference above a dialysis equilibrium in the manner of Hodgson.⁴ In using this formulation the activity coefficients of the ions in aqueous and the dialysate equilibrium fluid are assumed to be equal. This appears to be more reasonable than assuming identical activity coefficients in plasma and aqueous. The calculated energies for sodium and chloride can be converted to power in nanowatts by assuming an average flow of aqueous in the cat of $15 \mu\text{l}/\text{min}$. and using the watt-joule-calory equivalence relationships. The power in nanowatts can then be used to calculate the ion currents in μA by using the average membrane resistance of 47 ohms. Since the sodium ion current constitutes a positive current into the eye and chloride ion current a negative current in the same direction, the algebraic difference of the two would be the expected value of a measurement of the membrane current.

Using the aforementioned assumptions the net current was calculated to be approximately $23 \mu\text{A}$. The average membrane current of these experiments was $29.7 \mu\text{A}$. The sodium ion current based on these calculations is larger than the chloride ion current by this amount, and would cause a potential difference of about 1 millivolt with the neuroepithelial surface of the membrane negative with respect to the stromal surface. The potentials of these experiments averaged 1.2 millivolts with the same orientation of sign.

The implication of these speculations would be that the measured membrane current is possibly not due to sodium ion move-

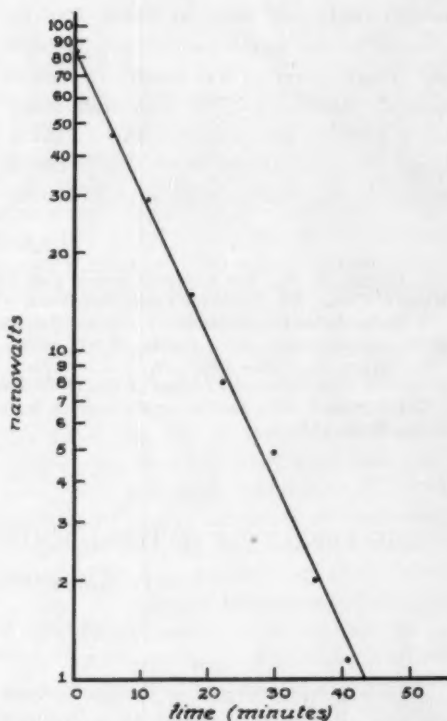


Fig. 5 (Holland Mallerich, Tischler and Belles-tri). Membrane power (E^2/R) appears to diminish as an exponential function of time.

ment alone, as it is in the frog skin, but to the algebraic difference of sodium and chloride ion currents. Whether this is the case could only be determined by further experimentation.

It is clear, however, that if the ciliary body does work in secreting aqueous humor, and if part of the work done consists in the movement of ions (as is currently presumed), then the electrical work calculated from the measurement of the membrane parameters should be related to this activity.

SUMMARY AND CONCLUSIONS

A high impedance, null-balance potentiometer in conjunction with a sensitive microammeter was used to measure the resistance of the cat iris-ciliary body, experimentally prepared as a membrane separating two res-

ervoirs containing identical fluids. The resistance of the membrane to both forward and reverse currents was measured, and its linearity examined. The measured membrane potential and resistance enabled calculation of the membrane short-circuit current as well as the electrical work done. The mem-

brane power was calculated and its possible correlation with secretory activity discussed.
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ACKNOWLEDGMENT

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THE EFFECT OF HYPOTHERMIA ON AQUEOUS HUMOR DYNAMICS

III. TURNOVER OF ASCORBATE AND SODIUM

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Systemic hypothermia in rabbits reduces the rate of aqueous secretion as determined by tonometric, tonographic and perfusion studies.¹ Flow declines exponentially with the decrease in body temperature. At 19°C secretion is estimated by these physical methods to be reduced to some 10 to 20 percent of the normothermic rate. It was the purpose of the present studies to measure the turnover of ascorbate and sodium in the aqueous humor of hypothermic rabbits in order to estimate the rate of aqueous flow.

METHODS

Hypothermia was induced in two to three kilogram male albino rabbits by immersion in an ice bath, using the short acting barbiturate, thiamylal sodium (Surital[†]), as necessary to prevent shivering during the induction period. Rectal temperatures were measured at regular intervals and room temperature was maintained at 20°C. Animals could be maintained at temperatures as low as 18-20°C for 5-8 hours.

The turnover of sodium²⁴ was carried out on 30 normothermic and 47 hypothermic animals at a mean (\pm S.D.) temperature of 19.2 (\pm 1.1)°C. Injections of approximately 50 μ c were made (one-third intravenously and two-thirds intraperitoneally so as to maintain a constant blood level). Samples of posterior chamber and anterior chamber aqueous humor as well as plasma were taken at various time intervals, air dried and counted in a proportional gas counter. Re-

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[†] Provided by Parke, Davis and Company.

sults were analyzed by linear (X-Y) methods.

The formulation used for these purposes was as follows:^{2,3}

$$\frac{dC_a}{dt} = k_{fa}C_h + k_{dpa}(C_p - C_a) - k_{fa}C_a$$

where

$\frac{dC_a}{dt}$ = the rate of change of concentration of the solute in the anterior chamber aqueous

k_{fa} = the rate of aqueous flow per minute as a fraction of the anterior chamber volume

k_{dpa} = the rate of diffusional exchange per minute of the solute between anterior chamber and plasma (as a fraction of the anterior chamber volume)

C_p = concentration in arterial plasma in relative units or mg percent

C_a = concentration in anterior chamber aqueous in relative units or mg percent

C_h = concentration in posterior chamber aqueous in relative units or mg percent

Dividing both sides of the equation by $(C_p - C_a)$:

$$\frac{dC_a}{dt} / (C_p - C_a) = k_{fa} \frac{(C_h - C_a)}{(C_p - C_a)} + k_{dpa}$$

where

$$Y = k_{fa}X + k_{dpa}$$

$$Y = \frac{dC_a}{dt} / (C_p - C_a)$$

$$X = \frac{C_h - C_a}{C_p - C_a}$$

Plotting X versus Y at different time intervals provided two separate straight lines corresponding to the normothermic and hypothermic data. For each of these lines the slope estimates k_{fa} and the Y-intercept k_{dpa} .

The turnover of ascorbate in 15 hypothermic animals ($19.8 \pm 0.9^\circ\text{C}$) was attempted at plasma levels of 10 mg percent by the administration of 50 mg of sodium ascorbate intravenously.⁴ When this proved unsuccessful as a method of measuring turnover, plasma levels averaging 160 mg percent were achieved in 60 hypothermic rabbits ($19.1 \pm 1.0^\circ\text{C}$) by the single intravenous injection of 1000 mg of sodium ascorbate. In addition 82 rabbits were "saturated" with ascorbate in the normothermic state by the administration of sodium ascorbate 100 mg intravenously and 100 mg subcutaneously. Two hours later plasma and aqueous samples from one eye of these normothermic rabbits were obtained. They were then subjected to hypothermia ($19.5 \pm 0.6^\circ\text{C}$) and the rates of change of ascorbate concentration in the posterior and anterior chambers were measured as functions of time. After hypothermia plasma levels were found to remain at approximately 10 mg percent in these experiments without need for further injection. Both of these ascorbate approaches provided data suitable for X-Y analysis. Ascorbate was determined on all samples by delivery into 4 percent metaphosphoric acid and titration immediately with dichlorophenolindophenol, using a 0.1 ml Gilmont ultramicroburet.

All posterior chamber and anterior chambers fluids were withdrawn from the proposed eyes into calibrated pipets. In hypothermic rabbits it was found necessary to use 27 gauge needles for posterior chamber taps in order to avoid vitreous contamination. Bloods were obtained by cardiac puncture using a heparinized syringe. Animals were used once and then discarded.

RESULTS

1. *Turnover of radioactive sodium.* The turnover of sodium²⁴ in the rabbit eye was plotted as a function of time (fig. 1). At each time interval the mean value \pm S.D. for 10 to 20 eyes was used. The values determined simultaneously in normothermic

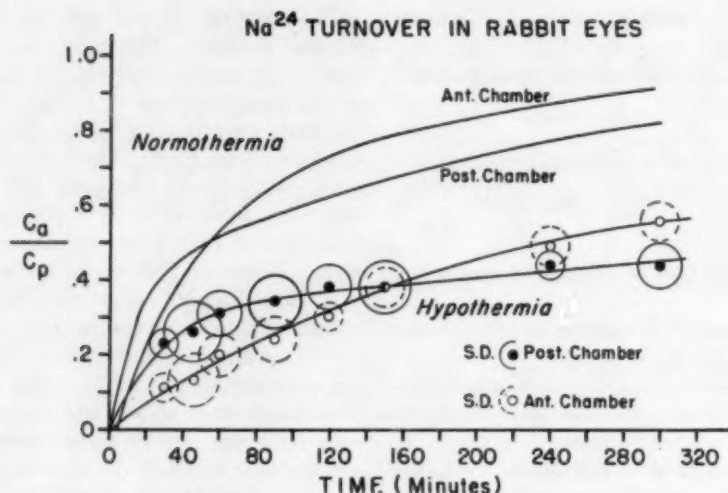


Fig. 1 (Becker). Relative concentrations of radioactive sodium in posterior and anterior chamber aqueous humors of hypothermic rabbits at various times after injection. At each time the mean value (\pm S.D.) for 10 to 20 animals is presented. Normothermic values drawn for comparison. C_a = concentration in aqueous humor, C_p = concentration in plasma water.

rabbits agreed well with those obtained previously. They are plotted in Figure 1 for comparison.⁸ The posterior and anterior chamber concentrations in the hypothermic animals at various times provided curves

that were of similar shape to that in the normothermic animals. It was apparent that the accumulation of radioactive sodium in both chambers of hypothermic animals was slower, and that the crossover point of the

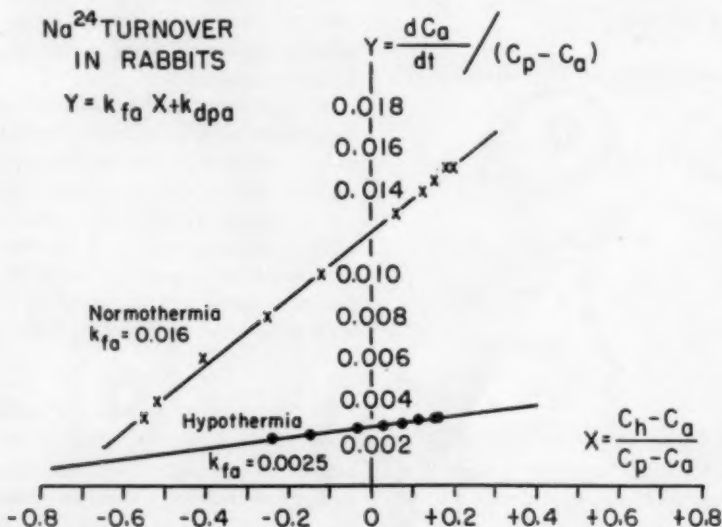


Fig. 2 (Becker). Linear (X-Y) analysis of sodium turnover in normothermic and hypothermic rabbits. The slope of each line estimates k_{fa} , the flow coefficient.

ASCORBATE TURNOVER IN RABBIT EYES

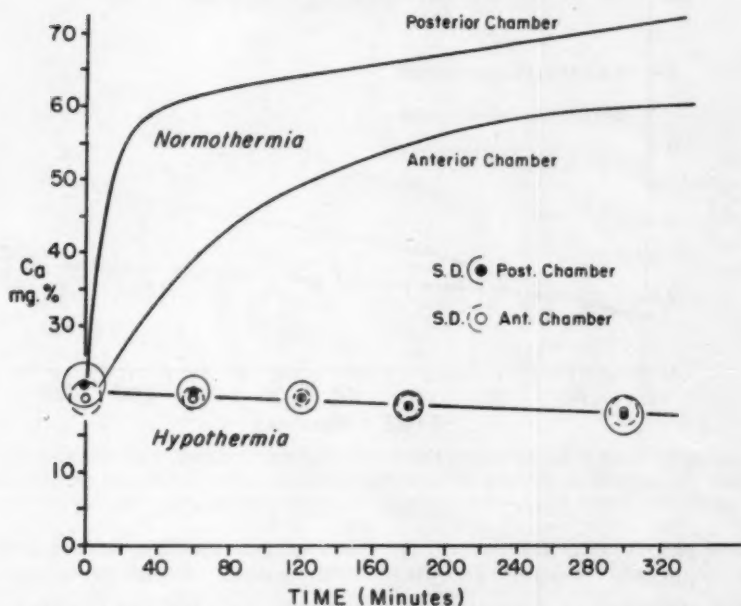


Fig. 3 (Becker). Concentration of ascorbate (C_a) in aqueous humor of posterior and anterior chambers of hypothermic rabbits at various times after the intravenous injection of sodium ascorbate. Plasma levels of 10 to 15 mg percent were maintained. The responses to this plasma level in normothermic rabbits are presented for comparison.

anterior and posterior chamber curves occurred later and at a lower concentration.

X-Y plots (fig. 2) of the posterior and anterior chamber data were analyzed and compared with normothermic rabbits. It was apparent that the rate of flow was reduced from the normal level of 1.6 percent of the anterior chamber per minute to 0.25 percent per minute at 19°C (a reduction to approximately 16 percent of the normothermic value). In addition the diffusion coefficient was decreased by hypothermia to 0.28 percent per minute from a normal of 1.2 percent per minute.

2. Turnover of ascorbate. In the normothermic animal the maintenance of a plasma concentration of 10 to 15 mg percent of ascorbic acid results in high concentration levels in the posterior and anterior chamber fluids. The rate of approach to these levels has been used as a means of measuring

aqueous flow.⁵ Attempts to utilize the same method in the hypothermic animal proved unsuccessful. In spite of a plasma level of 10 mg percent, posterior and anterior chamber ascorbic acid levels failed to rise, and in fact both declined slowly. Comparison of the normal and hypothermic responses to a plasma level of 10 to 15 mg percent of ascorbic acid are presented in Figure 3.

In order to alter aqueous ascorbate concentrations it was necessary to elevate the plasma ascorbate level considerably above the levels in the aqueous humor. This was accomplished by intravenous injection of 1000 mg of sodium ascorbate. In the hypothermic animals average blood levels of 160 mg percent resulted. Under these circumstances the concentrations in the posterior and anterior chamber aqueous humor rose slowly. In Figure 4 the aqueous humor concentrations for 10 to 20 eyes at each time

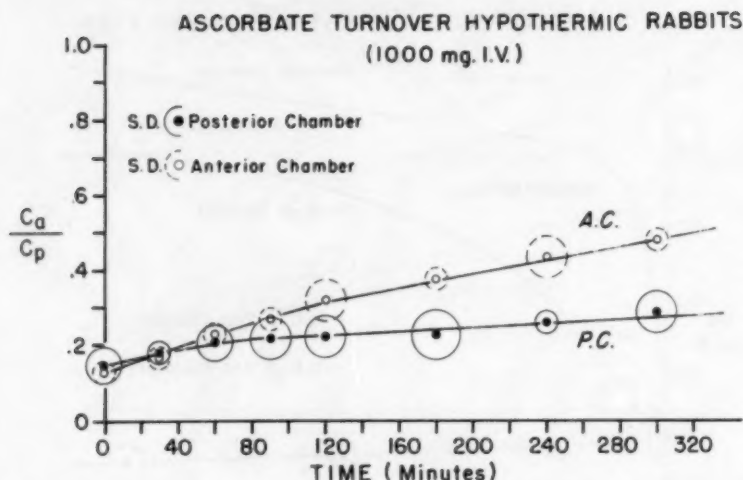


Fig. 4 (Becker). Aqueous humor concentrations of ascorbate in hypothermic rabbits at various time intervals after the intravenous injection of 1000 mg of sodium ascorbate. Plotted are the mean values (\pm S.D.) for aqueous humor (C_a) as a fraction of the plasma level (C_p) for 10 to 20 eyes at each time.

interval are plotted as a fraction of the plasma concentration (mean \pm S.D.) against time (minutes). It is apparent that the anterior chamber concentrations rose more rapidly than did those of the posterior chamber.

X-Y analysis (fig. 5) of the ascorbate

data suggested a flow rate of 0.20 percent of the anterior chamber per minute at 19°C. compared with previous estimates of 1.5 to 1.7 percent per minute in the normothermic animal (or a reduction to 12 to 13 percent of normal at this hypothermic level). The diffusion coefficient was reduced from the nor-

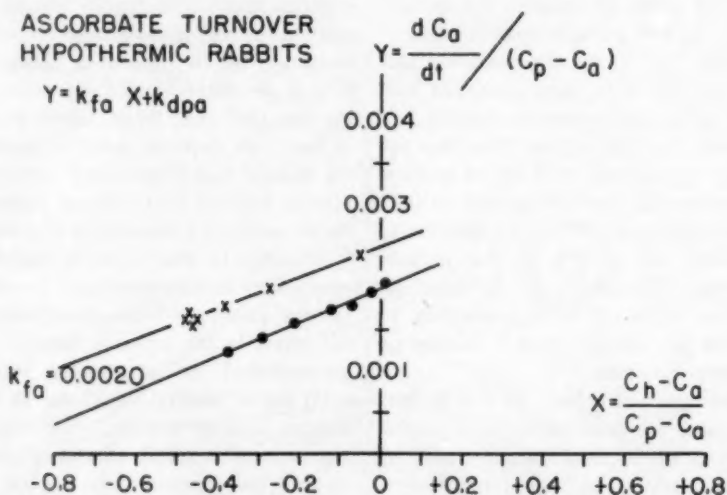


Fig. 5 (Becker). Linear (X-Y) analysis of ascorbate turnover in hypothermic rabbits. Circles represent data from intravenous injections of 1000 mg in hypothermic animals (see fig. 4); crosses are similar analysis of data of Figure 6. Slopes of lines provide estimates of k_{ts} , the flow coefficient.

ASCORBATE TURNOVER HYPOTHERMIC RABBITS
DECREASING CONC. AFTER SATURATION AT 40°C

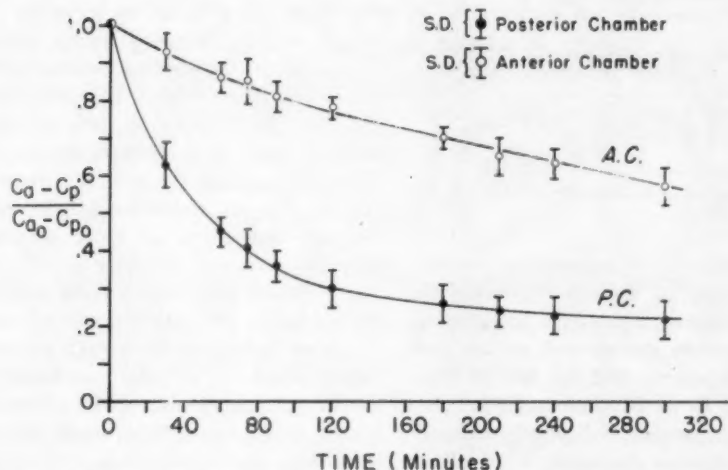


Fig. 6 (Becker). Rate of decline in aqueous humor ascorbate concentrations following hypothermia in the ascorbate saturated animal (sodium ascorbate 100 mg intravenously and 100 mg subcutaneously to normothermic animals two hours before hypothermia). Ascorbate excesses over plasma ($C_a - C_p$) for posterior chamber and anterior chamber are plotted as fractions of pre-hypothermic values (mean \pm S.D. for 10 to 15 eyes at each time interval).

mal of 0.5 percent per minute to 0.20 percent per minute at 19°C.

Another approach to ascorbate turnovers was provided by the administration of ascorbate 100 mg intravenously and 100 mg subcutaneously two hours before hypothermia was induced. This resulted in posterior chamber values averaging 66 percent, anterior chamber values of 50 percent, and plasma values of approximately 10 mg percent just prior to the time hypothermia was induced. One could then follow the time course of the fall in posterior and anterior chamber concentrations of ascorbic acid under hypothermia with a plasma level maintained at approximately 10 mg percent. These values were plotted as the mean (\pm S.D.) concentrations of ascorbate in excess of plasma in the aqueous humor of hypothermic (19.5°C) animals as a fraction of the values in excess of plasma before hypothermia (6 to 14 eyes at each time interval). This

method of plotting the data (fig. 6) tended to minimize the variation among animals and provided suitable curves for X-Y analysis. Under the circumstances of these experiments the posterior chamber concentrations fell much more rapidly than did those of the anterior chamber. This may well be related to the adjacent large volume of vitreous which had failed to become "saturated" in the brief two hour normothermic period. X-Y analysis of the data revealed a flow coefficient of 0.20 percent of the anterior chamber per minute and a diffusion coefficient of 0.25 percent of the anterior chamber per minute (fig. 5).

DISCUSSION

In Table 1 the results of the measurement of flow by various methods used on hypothermic rabbits are summarized and compared with the same techniques in normal and acetazolamide treated animals.⁶ For purposes of this comparison the volume of

TABLE 1
COMPARISON OF RATE OF AQUEOUS FLOW* IN
NORMAL, HYPOTHERMIC, AND ACETA-
ZOLAMIDE TREATED RABBITS

Method	Normal	Hypo- thermia (19°C)	Acetazol- amide
Ascorbate	4.3	0.5	2.1
Na ²⁴	4.0	0.6	1.8
Tonography	4.1	0.6	1.9

* Mean values ($\mu\text{l./min.}$) assuming volume anterior chamber of 250 $\mu\text{l.}$

the anterior chamber is assumed to average 250 μl and rate of flow is expressed in $\mu\text{l./min.}$ In animals at 19 to 20°C tonography as well as turnover studies with sodium and ascorbate demonstrate that the rate of flow is reduced to 10 to 20 percent of normal. Similar measurements following acetazolamide administration demonstrated that flow is reduced to 45 or 55 percent of normal. As in the case of carbonic anhydrase inhibition there is excellent agreement of the estimates of altered flow by diverse methods. In contrast to the acetazolamide effects which are confined to flow, however, hypothermia appears to also decrease the rate of diffusion of various test substances.

Unfortunately since flow and diffusion coefficients are both altered by hypothermia, it is not possible to use alterations in steady state concentrations as measures of change in flow rate for these animals. The use of this method presents further difficulties because of the enormous time interval needed to establish a new steady state when turnovers are so markedly reduced. There appears to be a tendency in the hypothermic rabbit, however, for the ascorbate concentration of the aqueous humor to fall and the bicarbonate concentration to rise.

Although it may be reasonably accepted that hypothermia decreases the rate of aqueous humor formation, the intimate mechanisms of this effect remain obscure. The decrease in aqueous flow may reflect a reduction in blood flow to the eye resulting from the decreases in cardiac output, pulse,

and blood pressure, the increase in viscosity of blood, and possible local vasoconstriction induced by cold. Unfortunately the marked suppression of secretion of ascorbate into the aqueous humor precludes the use of this method of estimating blood flow in the ciliary body.⁶ It is also conceivable that the decrease in rate of aqueous secretion may be a consequence of effects of hypothermia on the central nervous system, on various endocrine glands, on circulating metabolites, or more directly on the metabolism and secretory activity of the ciliary epithelium. Experimental approaches are needed to evaluate the role of each of the above factors in the overall process.

Alterations in diffusion coefficients for various substances also need further study. A part of the changes observed may result from such known effects of cooling as the reduction in rate of diffusion of solutes, alterations in solvent viscosity, and alterations in diameter of blood vessels. The temperature dependence of the metabolic state of living membranes and the active processes which maintain these barriers are also likely to be major factors in the alterations of the diffusion coefficients.

SUMMARY

In rabbits immersion hypothermia to 19°C has been reported to reduce the rate of aqueous flow to 10 to 20 percent of normal as measured by repeated tonometry and tonography. The turnovers of radioactive sodium and of ascorbate in the eyes of such hypothermic rabbits provided data compatible with this degree of secretory suppression. Thus, the coefficient of aqueous flow was reduced from the normothermic value of 1.6 percent of the anterior chamber per minute to 0.25 percent of the anterior chamber per minute at 19°C, as measured with sodium²⁴. The comparable values derived from ascorbic turnover were 1.7 percent normothermic and 0.20 percent at 19°C.

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THE EFFECT OF HYPOTHERMIA ON AQUEOUS HUMOR DYNAMICS

IV. CAROTID ARTERY LIGATION AND BLOOD FLOW*

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Aqueous flow is decreased following immersion hypothermia in rabbits.^{1,2} The present study attempts to determine whether there are changes in blood flow through the ciliary body during hypothermia and their possible relation to alterations in the rate of aqueous secretion. Unilateral carotid ligation and preganglionic sympathectomy are utilized in order to modify the alterations in ocular blood flow induced by hypothermia.

METHODS

The common carotid artery on one side was isolated, ligated, and cut under a short-

acting general anesthesia, thiamylal sodium,³ in 57 male albino rabbits weighing 2 to 3 kg. The contralateral eye was maintained as a control. Eighteen to 24 hours later immersion hypothermia was induced in an ice bath in the manner previously described.¹ Room temperature was maintained at approximately 20°C.

Tonography or tonometry was performed on both eyes prior to carotid ligation as a control. It was repeated 18 to 24 hours after the operation, before and during hypothermia. An electronic tonometer was used with a Leeds and Northrup recorder. Topical tetracaine (0.5 percent) was used for those rabbits studied at normal temperatures; however, it was not required for those animals studied under hypothermia. All scale readings were interpreted according to Wistrand's calibration curve for the rabbit.⁴ Aqueous flow rates were calculated from the equation $F = C(P_o - P_v)$ assuming an episcleral venous pressure of 9 mm Hg.

Unilateral preganglionic sympathectomy was performed on 12 rabbits. The effectiveness of the procedure was determined by

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³The thiamylal sodium (Surital sodium) was generously provided by Parke Davis.

the difference in ear temperatures. These rabbits were also studied by tonometry before and 18 to 24 hours after the operation and at intervals during hypothermia.

Blood flow through an inferior vortex vein was studied by a modification of the method of Linnér.⁴ The vein was isolated and incised with great care so as to prevent any extreme deviation of the globe. Following the parenteral injection of radioactive iodine (¹³¹I), the radioactivity in duplicate samples of blood collected on filter paper for exactly one minute each from a cut vortex vein was compared with the radioactivity found in 100 μ l of cardiac blood. Both experimental and control eyes of each rabbit were similarly approached and measured.

Rectal temperatures were measured with a deeply inserted laboratory thermometer. Temperatures in the anterior chamber and vitreous were determined by using a thermocouple (Waters Corporation) with a 24 gauge needle.

Crude relative estimates of ocular rigidity were obtained by comparing scale readings with 5.5 and 7.5 gram tonometric weights.

RESULTS

Intraocular pressure. The mean values for Schiötz readings and the corresponding intraocular pressures obtained during the progress of hypothermia are summarized in Table 1. Data thus obtained within 2°C of the tabulated temperatures were averaged for analysis. The intraocular pressure on the nonligated side decreased from a normothermic average of 18.5 mm Hg to 12 mm Hg at 22°C. At these corresponding temperatures the intraocular pressure on the ligated side fell from an average of 17.5 mm Hg to 10.0 mm Hg. When plotted as a logarithmic function the intraocular pressure on both sides fell exponentially with decline in rectal temperature (fig. 1). Intraocular pressure on the nonligated side fell 3.7 percent per degree centigrade decrease in body temperature. On the ligated side pressure decreased 3.9 percent per degree centigrade.

Outflow facility. Tonographic data from 20 rabbits before and approximately 24 hours after carotid ligation are tabulated in Table 2. In these rabbits the facility of out-

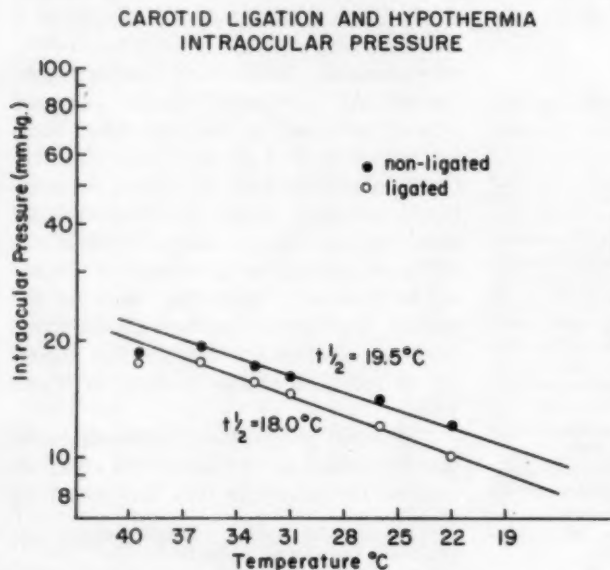


Fig. 1 (Pollack and Becker). Fall in intraocular pressure with decline in body temperature (semi-log plot). For every degree centigrade fall in temperature the pressure decreased 3.7 percent on the nonligated side (solid circles) and 3.9 percent on the ligated side (open circles).

TABLE 1
SUMMARY OF TONOMETRIC (SCHIÖTZ) DETERMINATIONS OF INTRAOCULAR
PRESSURE FOLLOWING* UNILATERAL CAROTID LIGATION

Temp °C	No. Rabbits	Scale Units		Intraocular Pressure		p(N.L. vs. L.)	p(vs. 39.5°C)	
		(Mean ± S.D.) N.L.	L.	(mm.Hg) N.L.	L.		N.L.	L.
39.5	37	4.5 ± 1.5	5.1 ± 1.6	18.5	17.5	> .05	—	—
36.0	11	4.2 ± 1.0	5.0 ± 1.1	19.0	17.5	.005	—	—
33.0	16	5.4 ± 1.0	6.6 ± 1.6	17.0	15.5	.005	.025	.005
31.0	22	6.1 ± 1.5	7.5 ± 1.7	16.0	14.5	.002	< .001	< .001
26.0	17	7.7 ± 1.5	10.4 ± 2.8	14.0	12.0	.002	< .001	< .001
22.0	18	10.0 ± 1.4	12.7 ± 2.6	12.0	10.0	< .001	< .001	< .001

N.L. = Nonligated side.

L. = Ligated side.

* 18-24 hours after ligation of common carotid artery.

flow on the ligated side was decreased significantly ($p < .001$) by an average of 16 percent. There was no significant change in facility of outflow on the nonligated side.

The above animals were then subjected to hypothermia and their body temperatures lowered to 22°C. Intraocular pressure fell with decline in body temperature in a manner similar to that described above during tonometric studies. As noted in previous studies¹ facility of outflow (C_H) decreased progressively with hypothermia when compared with the mean facility of outflow just before hypothermia was induced (C_N). This decrease occurred in similar fashion on ligated and nonligated sides and was inversely proportional to the change in viscosity of water associated with the lower temperatures (table 3).

Aqueous flow. The above tonometric and tonographic data demonstrated that aqueous flow, which had already decreased by 23 percent following carotid ligation, fell further during hypothermia (tables 1 and 2). It decreased exponentially by 12 percent per degree centigrade fall in rectal temperature beginning at approximately 36°C. On the nonligated sides of the same rabbits, aqueous flow began to decrease at about 35°C and

fell 11 percent per degree centigrade. At most hypothermic temperatures aqueous flow remained some 35-40 percent lower on the

TABLE 2
COMPARISON OF INTRAOCULAR PRESSURE AND FACILITY OF OUTFLOW IN NORMOTHERMIC RABBITS BEFORE AND AFTER CAROTID LIGATION AS DETERMINED BY TONOGRAPHY (20 RABBITS)*

	Nonligated Side	Ligated Side
P_1	18.0 ± 1.4	17.7 ± 1.6
C_1	0.32 ± 0.08	0.31 ± 0.06
P_2	17.8 ± 1.1	17.1 ± 1.5
C_2	0.31 ± 0.07	0.26 ± 0.06
$-\Delta F\%$	4 ± 13	23 ± 9
$-\Delta C\%$	3 ± 11	16 ± 10

* Mean ± S.D.

P_1 = Intraocular pressure before ligation (mm.Hg)

C_1 = Facility of outflow before ligation (μ l./min./mm.Hg)

P_2 = Intraocular pressure after ligation (mm.Hg)

C_2 = Facility of outflow after ligation (μ l./min./mm.Hg)

$-\Delta F\%$ = Percent decrease in aqueous flow

$$= 100 \frac{(P_1 - 9)C_1 - (P_2 - 9)C_2}{(P_1 - 9)C_1}$$

$-\Delta C\%$ = Percent decrease in facility of outflow

$$= 100 \frac{(C_1 - C_2)}{C_1}$$

TABLE 3
FACILITY OF OUTFLOW UNDER HYPOTHERMIA
FOLLOWING CAROTID LIGATION AS ESTI-
MATED BY TONOGRAPHY

	Nonligated Side	Ligated Side
C_N	$0.31 \pm .07$	$0.26 \pm .06$
C_H	$0.23 \pm .03$	$0.19 \pm .03$
C_{vis}	$0.33 \pm .04$	$0.28 \pm .04$

C_N = Facility of outflow (mean \pm S.D.) of 20 normothermic rabbits after ligation of the common carotid artery (from Table 2)

C_H = Facility of outflow (mean \pm S.D.) in hypothermic rabbits (22°C)

C_{vis} = Facility of outflow (mean \pm S.D.) in hypothermic rabbits adjusted to 39.5°C for viscosity of water

operated side than on the nonligated side (fig. 2).

Blood flow. When expressed as a percent of control values at normothermia on the nonligated side, the mean values for vortex vein blood flow fell exponentially with the decline in body temperature below 33°C. The decreases were 12 and 13 percent per degree centigrade on the nonligated and ligated sides, respectively (fig. 3). At 18°C blood flow was reduced to 15 percent of the nor-

mothermic rate on the nonligated side and to less than 10 percent on the ligated side. At any particular temperature during hypothermia the blood flow on the ligated side was approximately 50 to 55 percent that of the contralateral eye.

Ocular rigidity. The results of tonometric measurements with 5.5 and 7.5 gram weights are tabulated as apparent ocular rigidity coefficients in order to compare the findings on the ligated side with those of the nonligated side at both normothermic and hypothermic temperatures (table 4). No significant differences were noted between the apparent rigidity coefficients on the ligated and nonligated sides or between normothermic (39.5°C) and hypothermic (18°C) states.

Intraocular temperature. It was of particular interest to know how well the rectal temperatures correlated with the temperature inside the eye. At normothermic temperatures there were no significant differences between the intraocular temperatures measured on the carotid-ligated side as compared with those of the unoperated side. However, on both sides at normothermic body tem-

CAROTID LIGATION AND HYPOTHERMIA AQUEOUS FLOW

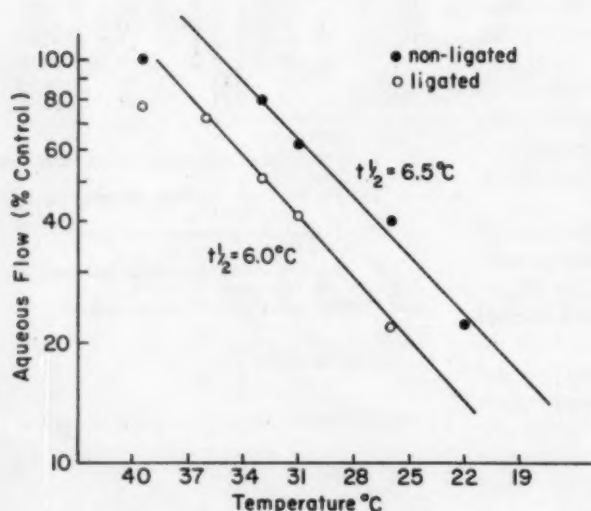


Fig. 2 (Pollack and Becker). Decline in rate of aqueous flow as determined by tonometry and tonography (expressed as a percent of the control value on the nonligated side) with decrease in rectal temperature. Aqueous flow decreases 11 percent per degree centigrade on the nonligated side (solid circles) and 12 percent per degree centigrade on the ligated side (open circles).

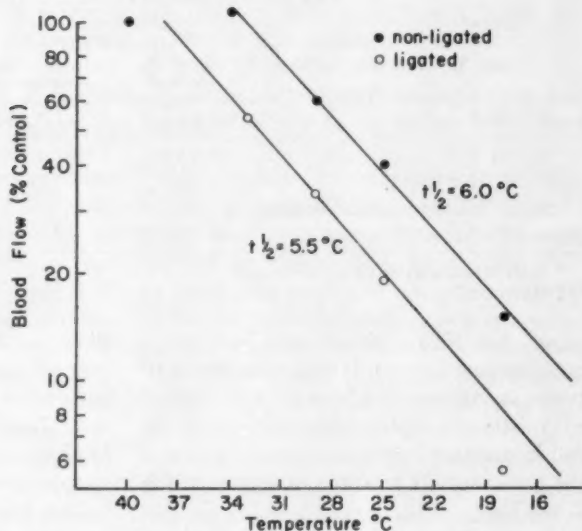
CAROTID LIGATION AND HYPOTHERMIA
BLOOD FLOW

Fig. 3 (Pollack and Becker). Decrease in rate of blood flow through one vortex vein (expressed as a percent of the control value on the non-ligated side) with decline in rectal temperature. Blood flow decreased 12 percent per degree centigrade on nonligated side (solid circles) and 13 percent per degree centigrade on the side of carotid ligation (open circles).

peratures the mean temperature of the anterior chamber averaged $1.97 \pm 0.82^{\circ}\text{C}$ * lower than that found in the vitreous and this difference proved significant ($p < .001$). In nine rabbits (18 eyes) the temperature in the anterior chamber and in the vitreous were determined during hypothermia and compared with the deep rectal temperature taken simultaneously. When the rectal temperature averaged 19.6 ± 1.8 , the temperature was 19.9 ± 1.6 in the anterior chamber and 19.8 ± 1.5 in the vitreous. During these experiments the mean room temperature was 22.1°C .

Preganglionic sympathectomy. The mean values for Schiøtz readings and the corresponding intraocular pressures obtained during the progress of hypothermia in rabbits with unilateral preganglionic sympathectomy are summarized in Table 5. It is evident that the intraocular pressure fell on the side of the sympathectomy in the same manner that it fell on the unoperated side as body temperature was lowered.

* Mean \pm S.D.

DISCUSSION

Eighteen to 24 hours after unilateral ligation of the common carotid artery in a total of 57 rabbits there is a small but statistically significant decrease in rate of aqueous secretion (by 20 to 25 percent). In many animals this is associated with a decrease in outflow facility, resulting in very little change in intraocular pressure. By tonography on anesthetized rabbits one to two days after ligation of the common carotid artery, Kornbluth and Linnér⁶ reported an intraocular pressure 3 mm Hg lower on the ligated side,

TABLE 4
SUMMARY OF APPARENT OCULAR RIGIDITY COEFFICIENTS BY SCHIÖTZ TONOMETRY AS DETERMINED WITH 5.5 AND 7.5 GRAM WEIGHTS

Temp. °C	No. Rabbits	Nonligated Side (mean \pm S.D.)	Ligated Side (mean \pm S.D.)
39.5	15	.014 \pm .005	.013 \pm .005
29.0	15	.014 \pm .005	.013 \pm .006
24.5	17	.014 \pm .009	.013 \pm .006
20.5	15	.012 \pm .005	.012 \pm .007
18.0	13	.011 \pm .006	.011 \pm .004

TABLE 5
SUMMARY OF TONOMETRIC (SCHIÖTZ) DETERMINATIONS OF INTRAOCULAR PRESSURE
FOLLOWING* PREGANGLIONIC SYMPATHECTOMY

Temp. °C	No. Rabbits	Scale Units†		Intraocular Pressure	
		Normal	Sympathectomy	Normal	Sympathectomy
39.5	8	4.2 ± 1.1	4.2 ± 1.4	19.0	19.0
31.0	8	5.6 ± 1.1	5.5 ± 0.9	16.5	16.5
25.0	6	7.3 ± 1.6	8.5 ± 1.9	14.5	13.5
22.0	8	8.7 ± 1.8	9.5 ± 3.1	13.0	12.5

* 18 to 24 hours after sympathectomy.

† Mean ± S.D.

using the 1955 Friedenwald calibration scale for human eyes. If pressures are interpreted using this Friedenwald scale instead of Wistrand's rabbit calibration curve, the fall in pressure following carotid ligation in the present study amounts to approximately 2 mm Hg.

As body temperature is lowered there is a progressive decrease in secretory rate on the ligated and nonligated sides. Both sides fall at similar exponential rates of approximately 11 percent per degree centigrade decrease in temperature so that the rate of secretion of aqueous humor on the ligated side remains approximately 35 to 40 percent below that of the contralateral eye during hypothermia.

As body temperature decreases the facility of outflow is lowered on both the ligated and nonligated sides (table 3). It has been shown that the decrease in facility of outflow that occurs during hypothermia can be accounted for in large part by the increased viscosity of aqueous humor.³ It is apparent that this same conclusion applies to both eyes of the rabbit previously subjected to unilateral carotid ligation. Thus, if facility coefficients under hypothermia (C_H) are adjusted for viscosity to 39.5°C (C_{v18}), the values on the ligated and nonligated sides compare favorably with those before hypothermia (C_N).

As a consequence of the decrease in aqueous secretion and the symmetrical change in outflow facility, the intraocular pressure de-

clines on both sides at a very similar exponential rate of approximately 3.8 percent per degree centigrade fall in body temperature. Thus intraocular pressure is some 10 to 15 percent lower on the ligated side at all temperatures.

The finding of no significant change in apparent ocular rigidity as estimated with two tonometric weights is reassuring but by no means conclusive. The excellent agreements of aqueous flow changes during hypothermia as calculated from tonometry and turnover studies also suggests that ocular rigidity is altered very little.

The present decrease in aqueous flow (20 to 25 percent) observed 18 to 24 hours after carotid ligation is considerably less than the 45 percent decrease reported by Kornbluth and Linnér.⁵ This discrepancy is partly a consequence of the difference in calibration tables, but may also be related to the general anesthesia used for tonography by these authors. The present values resemble closely the 30 percent lower flow on the ligated side estimated by comparing fluorescein appearance time in the two eyes.⁵ It is similar, also, to the decrease calculated from ascorbate steady state levels (15 to 20 percent) and from isotope turnover studies (20 to 25 percent).

It is of particular interest to compare the magnitude of the decrease in aqueous flow and the estimated effect of carotid ligation on ciliary body blood flow. Thus Linnér⁴ found that blood flow, as measured from the

cut vortex vein, was reduced 19 percent, 18 to 24 hours after carotid ligation. He also reported that unilateral ligation of the common carotid artery in rabbits reduced the concentration of ascorbate in the anterior chamber by approximately 17 percent.⁶ The similarity of these two figures provided evidence in favor of the hypothesis that anterior chamber aqueous ascorbate concentrations could be used to measure ciliary body blood flow. However the 17 percent figure assumes no change in aqueous flow or in diffusion out of the anterior chamber on the ligated side. If the secretory rate is reduced by as much as Linnér estimates (45 percent), then ciliary blood flow is decreased by some 55 percent on the side of carotid ligation. More accurate estimates of ascorbate transfer can be obtained from posterior chamber aqueous humor data. Here the ascorbate concentration on the ligated side averages 86 percent of the values for the contralateral eye. If this value is corrected for the current estimates of secretory depression on the ligated side, ciliary body blood flow may be reduced by as much as 30 to 40 percent following carotid occlusion. This estimate assumes no alteration in the ascorbate secretory capacity of the ciliary epithelium or in the diffusion characteristics of the membranes surrounding the posterior and anterior chambers. It is difficult to reconcile these estimates with the 19 percent figure obtained by Linnér on cutting the vortex vein.

In the present studies it has been demonstrated that vortex vein blood flow, as estimated, declines exponentially with body temperature. Of particular interest is the marked similarity in the exponential rates of fall for blood flow and for aqueous secretion as body temperature is lowered. Furthermore blood flow on the nonligated side does not begin to fall measurably until temperature is lowered to 33 to 35°C—a delay similar to that found for the effects on aqueous secretion and intraocular pressure. When the body temperature is cooled to approximately 19°C, both aqueous flow^{1,2} and blood flow (figs. 2

and 3) are reduced to 10 to 20 percent of their normothermic rates. Similarly, on the side of the carotid ligation, vortex vein blood flow decreases with hypothermia at an exponential rate of 13 percent per degree centigrade, a rate very similar to the nonligated side and to the decline in aqueous secretion.

The findings are suggestive of a direct causal relationship between the fall in ocular blood flow associated with hypothermia and the decline in aqueous secretion. The lower secretory rate at all temperatures on the side of carotid ligation also suggests a relation of aqueous secretion to ciliary body blood flow. Blood flow on the ligated side during hypothermia remains some 50 to 55 percent of the nonligated side, and values for aqueous flow are approximately 60 to 65 percent of the contralateral eye. The small difference may represent a certain reserve capacity of the secretory process or may well result from errors of measurement. The similar anterior chamber temperatures in spite of the differences in rate of aqueous flow on ligated and nonligated sides also suggest blood flow rather than ciliary body temperature as a major factor. However, multiple components may be involved in the effects of carotid ligation on aqueous secretion. These may be independent of an additive to the effects of hypothermia, without any reference to blood flow.

The possibilities must be borne in mind that both changes in blood flow and secretion may be consequences of some common factor or that vortex vein blood flow may even be influenced by intraocular pressure. Furthermore, the relative changes in blood flow as determined in a single vortex vein do not necessarily reflect the changes in blood flow to the ciliary body. Finally, one cannot disregard the possibilities of some more direct effects of cooling on the metabolic and functional status of the secretory cell.

Several closely associated physiologic changes may contribute to the decline in

ocular blood flow during hypothermia. These include a fall in pulse, cardiac output and blood pressure, and a rise in peripheral vascular resistance, hematocrit, and blood viscosity. At 19°C vortex vein blood flow is decreased by 80 to 90 percent. Similar decreases in carotid blood flow in cats have been reported by Stern and Good.⁷ They noted a decline of 9 percent per degree centigrade so that at about 19°C blood flow was 80 to 90 percent lower than the pre-cooling baseline. A similar but smaller fall has been noted for renal⁸ and cerebral blood flow.⁹

The failure of preganglionic sympathectomy to alter the effects of hypothermia on aqueous humor dynamics suggest a minor role of ocular vasoconstriction in the changes in blood flow and aqueous secretion.

SUMMARY

Following unilateral carotid artery ligation in the rabbit, immersion hypothermia resulted in decreases in rate of secretion of aqueous humor in both eyes. Comparative tonometric and tonographic studies suggested an exponential fall on both sides of 11 to 12 percent per degree centigrade decline in rectal temperature. At hypothermic temperatures aqueous flow remained approximately 35 to 40 percent lower on the operated side than on the nonligated side.

Intraocular pressure also decreased exponentially on both ligated (3.9 percent per degree centigrade) and nonligated sides (3.7 percent per degree centigrade). Ocular rigidity, as measured with 5.5 and 7.5 gram

weights, remained unchanged following carotid ligation and during hypothermia.

During hypothermia no statistical differences were found between the rectal and intraocular temperatures nor did the carotid-ligated and nonligated sides differ in temperature. At normal body temperature, however, the mean temperature of the anterior chamber was about 2°C lower than that found in the vitreous or by rectal measurement.

Unilateral preganglionic sympathectomy did not change intraocular pressure or alter the effects on intraocular pressure of lowering body temperature as compared with the unoperated side.

Ocular blood flow, as measured from a cut vortex vein, fell exponentially with the decline in body temperature in a very similar fashion to the decrease in aqueous secretion. Following unilateral carotid ligation both homolateral and contralateral eyes demonstrated 12 to 13 percent decreases in blood flow per degree centigrade fall in body temperature. At hypothermic temperatures vortex vein blood flow remained approximately 50 to 55 percent lower on the ligated side than in the opposite eye. This suggested a possible role of ciliary body blood flow as a factor in the effects of hypothermia on aqueous secretion.

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THE TRANSPORT OF ORGANIC ANIONS BY THE RABBIT CILIARY BODY

IV. ACETAZOLAMIDE AND RATE OF AQUEOUS FLOW

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Iodopyracet and related organic anions are secreted out of the rabbit eye.^{1,2} Intravitreal trace doses of iodopyracet are rapidly cleared from the eye by this transport system without gaining access to the anterior chamber. Injections into the vitreous humor of doses of iodopyracet which exceed the maximum transport capacity produce accumulation in the anterior chamber and exit from that chamber by flow and diffusion.

Iodomethamate (Neo-iopax) is one of the related organic anions secreted out of the eye by a mechanism similar to that for iodopyracet. The rate of transport of iodomethamate is somewhat slower, but nevertheless trace doses do not gain access to the anterior chamber. Saturation kinetics and inhibition by probenecid, iodopyracet, penicillin, and paraaminophenylacetate are also essential features of iodomethamate transport.

Systemic acetazolamide in quantities sufficient to reduce aqueous flow substantially, does *not* alter the total rate of loss from the rabbit eye of trace, intermediate, and saturating doses of iodopyracet or iodomethamate. This paper reports an investigation of the effect of systemic acetazolamide on the anterior chamber accumulation of these organic anions in the saturated transport system. The results provide an independent measurement of the alteration of aqueous humor secretion produced by acetazolamide.

METHODS

Each of ten albino rabbits (approximately two kg) received an injection of 20 microliters of a solution containing approximately 10.5 mg nonlabelled iodopyracet and 0.5 to 1.0 microcuries of ¹³¹I-labelled iodopyracet† into the vitreous humor of the right eye. Rabbits number 1 to 5 received 100 mg/kg acetazolamide intravenously just prior to the intravitreal injection and 50 mg/kg acetazolamide intraperitoneally every hour thereafter. Seven hours after the intravitreal injections the right eyes were counted by means of a scintillation well counter (model DS3—

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† Abbott Radio-Diadrast sterile solution.

Nuclear Chicago) turned on its side and used as an external probe. All ten right anterior chambers were then tapped and the aqueous humor samples assayed in the well of the same scintillation counter. Two days later the same procedure was followed using the left eyes of the same ten animals and administering acetazolamide to numbers six to ten.

The same experiment was performed on another group of ten albino rabbits using for intravitreal injection 20 microliters of a solution containing approximately 7.5 mg of nonlabelled iodomethamate and 0.5 to 1.0 microcuries of I^{131} -iodomethamate.*

MATHEMATICAL FORMULATION

The rate of loss of iodopyracet from the eye is the sum of the rate of transport out of the eye behind the iris and the rate of loss from the anterior chamber:³

$$\frac{dI}{dt} = v + k_a I_a = v + k_o r I_v \quad (1)$$

where

$\frac{dI}{dt}$ = the amount of iodopyracet leaving the eye per minute

I = the amount of iodopyracet in the eye ($= I_a + I_v$)

I_a = the amount of iodopyracet in the anterior chamber

I_v = the amount of iodopyracet in the vitreous humor (approximately the same as I)

v = the amount of iodopyracet secreted out of the eye per minute

k_o = the turnover coefficient of iodopyracet in the anterior chamber or the fraction of iodopyracet in the anterior chamber leaving per minute (by flow and diffusion)

$r = I_a/I_v$, the anterior chamber accumulation ratio

Let the superscript (D) signify that the turnover of iodopyracet in the vitreous occurred under the influence of systemic

acetazolamide. Each animal had one eye for which this was the case. The following equations relate iodopyracet turnover between the acetazolamide-treated and non-treated eyes of each animal. Acetazolamide did not alter either the total amount of iodopyracet lost from the eye per minute

$\left(\frac{dI}{dt}\right)$ or the amount per minute secreted

out of the eye (v):

$$\frac{dI^{(D)}}{dt} = \frac{dI}{dt}$$

$$v^{(D)} = v$$

$$I_v^{(D)} = I_v$$

Therefore $k_o^{(D)} r^{(D)} = k_o r$ and

$$\frac{k_o^{(D)}}{k_o} = \frac{r}{r^{(D)}} \quad (2)$$

or the turnover coefficients vary inversely as the accumulation ratios in the anterior chamber.

These equations are applicable in similar fashion to the iodomethamate turnover studies.

RESULTS

The ratios $r^{(D)}/r$ are tabulated for both series of experiments (Tables I and II). Acetazolamide produced a mean increase of 66 percent in the iodopyracet anterior cham-

TABLE I

EFFECT OF ACETAZOLAMIDE ON THE ANTERIOR CHAMBER ACCUMULATION RATIO OF IODOPYRACET

Rabbit No.	$r^{(D)}/r$
1	1.56
2	1.79
3	1.89
4	1.70
5	1.61
6	1.67
7	1.89
8	1.47
9	1.64
10	1.37
Mean 1.66 ± 0.17 (S.D.)	

$r = I_a/I_v$, the anterior chamber accumulation ratio in the untreated rabbit.

$r^{(D)}$ = the anterior chamber accumulation ratio in the acetazolamide-treated rabbit.

* Prepared by Abbott Laboratories.

TABLE II

EFFECT OF ACETAZOLAMIDE ON THE ANTERIOR CHAMBER ACCUMULATION RATIO OF IODOMETHAMATE

Rabbit No.	$r^{(D)}/r$
11	1.61
12	1.75
13	2.13
14	1.67
15	1.61
16	2.04
17	1.61
18	1.85
19	1.67
20	1.54
Mean 1.75 ± 0.20 (S.D.)	

$r = I_a/I_v$, the anterior chamber accumulation ratio in the untreated rabbit.

$r^{(D)}$ = the anterior chamber accumulation ratio in the acetazolamide-treated rabbit.

ber accumulation ratio. This corresponds to a decrease in the turnover coefficient, k_0 , for iodoxypracet to 60 percent of the control value following acetazolamide administration. The results of similar studies with iodomethamate revealed a mean increase of 75 percent in the accumulation ratio or a decrease in k_0 to 57 percent of the control values.

DISCUSSION

The method employed in these studies requires an accurate evaluation of the effects of acetazolamide on the behavior of the two organic anions under conditions of saturation. Using one eye of the rabbit as a control for the acetazolamide-influenced eye tends to minimize the effect of individual animal variation. This was further controlled by administering acetazolamide in connection with the first intravitreal injection for half

of the animals and in connection with the second injection for the other half. Furthermore, this type of intravitreal injection of organic anions has been demonstrated not to alter significantly either tonography or anterior chamber chemistries.¹

The computation is based upon the findings that the active transport process out of the eye for the two organic anions as well as their movements within the eye are not altered significantly by acetazolamide. Thus the rate of entry into the anterior chamber from behind the pupil is the same in the treated and control eyes. At a given rate of entry into the anterior chamber, a substance accumulates to attain that concentration at steady state for which rate of loss balances the rate of entrance. Since the total amount lost per minute remains the same, the concentration in the anterior chamber varies inversely with the turnover coefficient, k_0 , for that substance. Therefore, the measurement of anterior chamber accumulation at steady state provides an accurate index of k_0 (figs. 1 and 2).

Following systemic administration of these organic anions to the nephrectomized rabbit, anterior chamber penetration does occur, but there is virtual exclusion from the vitreous humor¹ and posterior chamber.⁴ Under steady state conditions the ratio C_a/C_p of anterior chamber concentration to plasma concentration was found to be 0.16 for iodoxypracet and 0.14 for iodomethamate.

Since entry of these substances into the anterior chamber is by diffusion and loss from the anterior chamber occurs by flow

IODOPYRACET - SATURATION - NORMAL

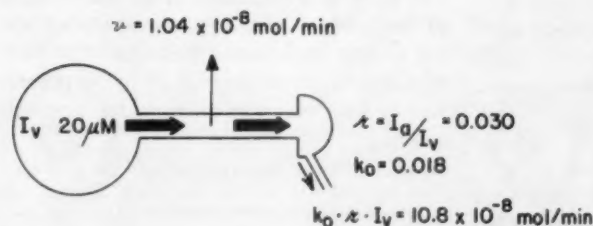


Fig. 1 (Forbes and Becker). Schematic representation of mechanism of turnover of an intravitreal saturating dose ($20 \mu\text{M}$) of iodoxypracet. The secretory rate (v), anterior chamber accumulation ratio (r), and rate of loss through the anterior chamber ($k_0 r I_v$) are shown.

IODOPYRACET-SATURATION-ACETAZOLAMIDE

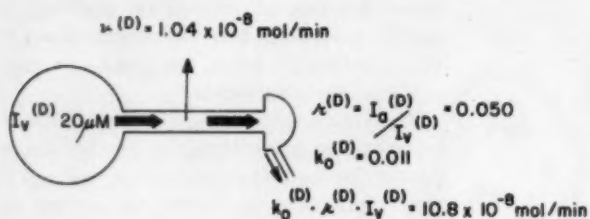


Fig. 2 (Forbes and Becker). Schematic representation of mechanism of turnover of an intravitreal saturating dose ($20 \mu\text{M}$) of iodopyracet under the influence of acetazolamide. The secretory process ($v^{(D)}$) is not altered. There is a 40 percent reduction in the exit rate from the anterior chamber ($k_o^{(D)}$) resulting in a 66 percent increase in the anterior chamber accumulation ratio ($r^{(D)}$). The rate of loss through the anterior chamber ($k_o^{(D)} I_o^{(D)}$) is not altered.

and diffusion:

$$\frac{dC_a}{dt} = k_d(C_p - C_a) - k_f C_a$$

where

k_d = rate of diffusional exchange per minute between anterior chamber and plasma as a fraction of anterior chamber volume

k_f = rate of aqueous flow per minute as a fraction of anterior chamber volume

k_o = fraction of substance in anterior chamber lost per minute = $k_d + k_f$

At steady state:

$$\frac{dC_a}{dt} = 0$$

and:

$$k_d = \frac{k_f C_a}{C_p - C_a}$$

Assuming that acetazolamide does not alter k_d , the diffusion coefficient for these large anions, the decreases in k_o values for the anterior chamber found in the present studies can be used to calculate the reduction in k_f , the rate of flow of the aqueous humor. For iodopyracet:

$$\begin{aligned} k_d &= 0.19 k_f = k_d^{(D)} \\ \frac{k_o^{(D)}}{k_o} &= \frac{k_f^{(D)} + k_d^{(D)}}{k_f + k_d} = 0.60 \\ \frac{k_f^{(D)}}{k_f} &= 0.52 \end{aligned}$$

A similar computation for the iodomethamate data yields the ratio $k_f^{(D)}/k_f = 0.50$. Therefore, the measured increase in anterior

chamber accumulation ratios for these organic anions produced by acetazolamide corresponds to average decreases in the rate of secretion of aqueous humor of 48 to 50 percent.

Previous estimates of the reduction in aqueous humor flow in rabbits produced by acetazolamide were based upon tonographic measurements and aqueous humor turnover studies.⁵ It is of interest that the present results obtained by an independent method are in close agreement with previous determinations.

SUMMARY

Following the injection of a saturating dose of either iodopyracet or iodomethamate into the rabbit vitreous humor, there was anterior chamber accumulation with consequent exit from the eye via the flow-diffusion mechanism. Systemic administration of acetazolamide altered neither the transport process for these organic anions nor the total amount of either lost from the eye per minute. Acetazolamide did produce average increases of 66 and 75 percent respectively in the anterior chamber accumulation ratios of the two anions. These corresponded to 40 and 43 percent decreases in k_o , the fraction of the anterior chamber concentrations lost per minute by flow and diffusion. The findings were compatible with 48 to 50 percent suppression of aqueous flow by acetazolamide.

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ACKNOWLEDGMENT

The authors acknowledge gratefully the technical assistance of Mrs. Tommie Tracy and Miss Carol Fritz.

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MORPHOLOGY OF THE CORNEA

I. STUDY WITH SILVER STAINS

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The structure of the cornea has interested many investigators beginning in last century.^{1,2,3,4,5,6,7,8} Many methods and techniques were used to determine the arrangement of the corneal fibres in addition to the usual histologic techniques. Recently, investigations with polarized light,^{9,10,11,12} and with the electron microscope,^{13,14} have added much important information on the arrangement of the collagen fibres. For the most part these recent studies have confirmed the earlier investigations which described a system of fibre bundles in the cornea.

The present report is concerned with analyzing the corneal structure as revealed with the Silver Carbonate of del Rio Hortega. Brief comparison of the fibre arrangement in the mammalian corneas with that found in other species and in young animals, also, is made. The arrangement of corneal fibres is of importance in understanding its structure in relation to surgical procedures which necessitate dissection of corneal lamellae, and in understanding the optical properties of the cornea.

MATERIAL AND METHODS

Normal corneas of young and adult rabbits, human, beef and dog were studied. Comparisons were made with cornea of chicken, and with two species of fish (Teleost and Elasmobranch). The corneas were excised and fixed in 10 percent Formalin (pH 7.2, or pH 4.5). After washing in tap water for several hours to remove the fixative, they were stained in toto in Silver Carbonate of del Rio Hortega¹⁷ medium strength solution, pH 12 at 55-60 C from three to four hours. Following the staining procedure, small radial cuts were made in the corneas so they could be placed as nearly flat as possible in the freezing microtome. In the mammalian corneas wrinkles were not observed, but the dome of the cornea could not be flattened completely. Tangential flat sections of about 10 μ thickness were made. Some stained rabbit corneas were mounted in a board and careful lamellar dissections were made in the manner commonly used to dissect superficial layers of the cornea in surgical procedures.

RESULTS AND COMMENTS

When removing the superficial lamellae of the cornea one could sense that the knife was cutting through a palisade of corneal fibres, even though a good lamellar dissection was

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being obtained. If the lamella was lifted from the underlying stroma and examined with a loupe, a vertical palisade of fibres interweaving in all directions was observed connecting the underlying stroma with the superficial layers being dissected. If such dissection was made of the most superficial layers it was not found possible to obtain good cleavage of the corneal tissue into lamella. The flat sections made parallel at 15 to 20 μ below the epithelium shows that the fibres appeared to form a tight interlacing meshwork which would obviously explain the difficulty in splitting this layer into lamellae. This arrangement is also compatible with clinical observations that edema of the superficial layers of the cornea is infrequent.^{9a}

When slightly deeper sections are examined, one can see that fibre bundles are present but not perfectly delineated. Sections taken through the midstroma show neat and sharply defined bundles or ribbons of fibres. These ribbons of corneal fibres cross each other at many different angles, and only occasionally at right angles. At the same

time they appear to interweave with one another so that they shift from one antero-posterior level to another as if they were in fact woven as in a fabric (Fig. 1 and 2). According to Salzman⁷ these fibres weave in and out at very small angles to each other so that their course is almost always parallel to the surface. At times these bundles branch out in two or three subsidiary branches or bundles. Complete reconstruction of the cornea which would allow the tracing of the ribbons from their origin to their destination has not been possible in our frozen sections.

However some bundles seem to progress from one limbic area completely across the cornea to the opposite limbus, while other bundles travel upwards to end in the sub-epithelial zone of the cornea. In transverse, vertical or horizontal sections, it is impossible to observe the intercrossing of these fibers, except in swollen corneas. This is demonstrated in Figure 3 in which one can see the schematic trajet of the bundles of fibres. These bundles can be called elementary lamellae since a lamella as seen in a transverse section is actually formed by a

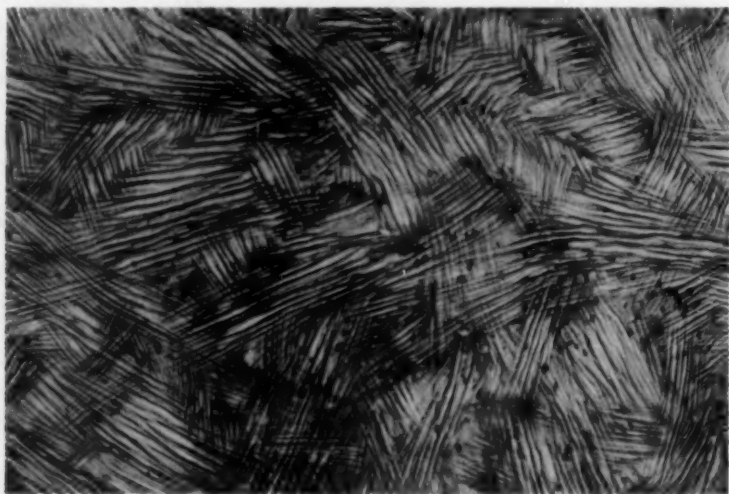


Fig. 1 (Polack). Flat tangential section of rabbit cornea through the mid-stroma showing bundles of fibres interweaving with one another. A similar aspect is found in deeper layers. Silver Carbonate of Rio Hortege. Microphotograph 7 \times .



Fig. 2 (Polack). An enlarged area of the preceding photograph to show the bundles of argyrophillic fibres. Silver Carbonate of Rio Hortege, 86 \times .

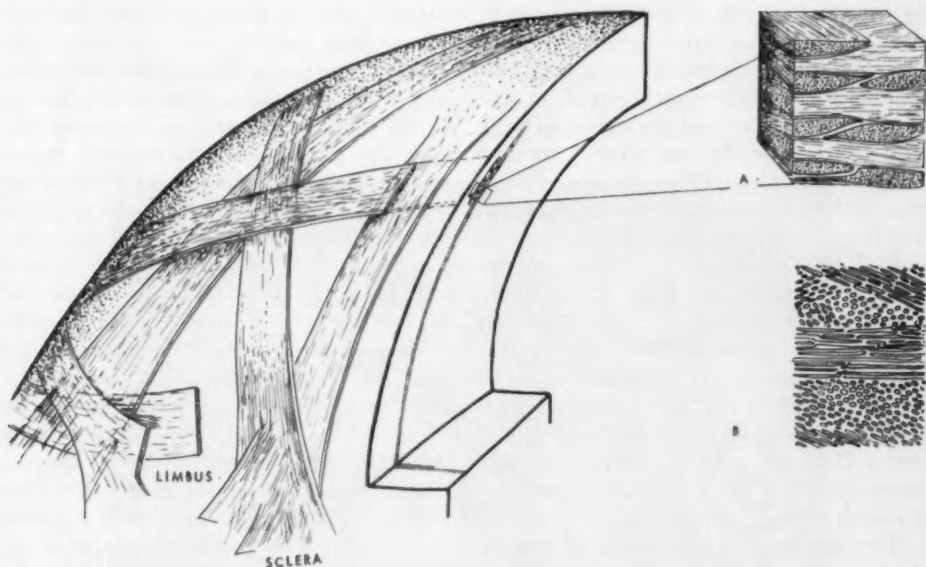


Fig. 3 (Polack). Scheme showing the possible arrangement of the collagen bundles in one lamella. A horizontal and a vertical section has been made to demonstrate the appearance of layers parallel to the surface in these sections.

In the insert A, the arrangement of the fibres in the anteroposterior direction is represented.

In B, the arrangement of the fibrils inside the fibres is represented as seen with the Electron microscope. (This drawing was obtained from an electron micrograph of Jakus from a rat cornea.)



Fig. 4 (Polack). Peripheral section of the cornea showing bundles of fibres arranged circularly (C). The limbic area is in the lower part of the picture. Some bundles cross these circular fibres at right angles while others do so at oblique angle (O). Silver Carbonate of Rio Hortega. 86X.

reunion of interwoven fibre bundles which have a displacement of several microns in the anteroposterior axis. It is particularly this antero-posterior intermingling that holds together the several corneal layers. Examination of the periphery of the cornea in these sections through the mid-stroma revealed some circular fibres (Fig. 4). The orientation of these corneal bundles with respect to the insertion of the recti muscles as described by Kokott in human corneas⁹ was not observed in our preparations.

At higher magnification it can be seen that the fibres are not round but flat (Fig. 5); these observations are in agreement with those obtained in electron microscope studies.^{15,16} In these studies it has been noticed that a fibre is formed by grouping together numerous fibrils all traveling in the same direction within the given fibre.

The pattern of the fibre bundles in the deeper layers of the cornea does not seem to differ from that in the middle layers, except at the periphery where they spread out fan-wise intermingling with the scleral fibres and crossing some circular fibres, at this level, a

ring of circular fibres such as described by Kokott,⁹ and suggested by Pratt-Johnson¹⁰ has not been found.

It is interesting to observe that the organization of these fibres, very similar to that of the adult, was present in the young rabbits. The pattern of fibres reported in the cornea of the adult rabbit was found to be the same in the human and in the dog cornea. Corneas of Scup, dogfish and shark also were investigated; staining of these corneas was quite difficult, perhaps because the amount of mucine present, however it could be demonstrated that their corneas also were made of fibre bundles. These bundles or ribbons appeared to be more uniform in width and the crossings were nearly at right angles, this was observed best in the Elasmobranch corneas in which species the fibre bundles were found to be slightly displaced in successive layers so they appeared to rotate around the antero-posterior axis of the cornea when lamella or strata situated at different depths were observed.

This rotation of successive lamellae resembles that reported by Coulombre in the

chick.¹⁴ The observations reported here are in agreement with those of Virchow² early in this century, who showed a pattern of fibres in corneas of several species by interstitial injections of mercury and dyes. Similar observations to those of Virchow were reported later by Ehlers,⁵ Krauss,⁶ and Kokott.⁸ According to Maurice¹⁰ and Kikkawa^{12a,d} the structure of the corneal collagen and the arrangement of its fibres is of great importance in relation to its transparency. The concepts of Maurice and Kikkawa are based on the ultramicroscopic structure of the collagen bundles and the fibre arrangement. Studies with polarized light suggested that the cornea fibres are radially arranged,^{9, 10, 11, 12, 13} however Kikkawa^{12a} says that except for the work of Kokott there was no anatomical demonstration that the corneal fibres were in fact arranged radially. Stanworth and Naylor^{10a} and Kikkawa^{12b} attempted to obtain the probable pattern of collagen fibres by deducing it from the optical properties of the cornea. Naylor^{11b} using polarized light found that in addition to the radial arrangement of the fibres, cross-linking occurs between strata so that

the direction in which the fibres run in successive lamella are random with respect to the cornea as a whole. Similar observations were made by Mishima.¹³ This is in agreement with the results reported here and which are offered as the anatomical demonstration that the fibres do in fact fit the pattern suggested by the studies with polarized light.

The fibre bundles in the mammalian cornea appear to be distributed in a similar fashion throughout most of the corneal stroma. This is based primarily on studies in the rabbit cornea, but preparations of human, beef and dog corneas are in agreement with it. The rotation of the pattern of bundles as seen by Coulombre in the chick could not be demonstrated in the mammalian cornea, although evidence that this occurs in more primitive species was obtained.

SUMMARY

The structure of the collagen fibres in the rabbit cornea has been studied with the Silver Carbonate of del Rio Hortega. It has been shown that there is a network of bundles of argyrophillic fibres crisscrossing and

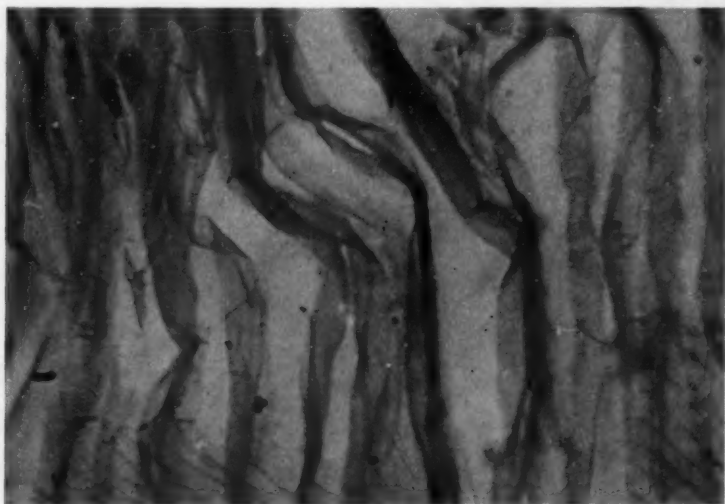


Fig. 5 (Polack). Microphotograph showing the flattened structure of the fibres. Silver Carbonate of Rio Hortega, 280 \times .

interweaving with each other at various random angles. It is suggested that this picture is in agreement with the observations by polarized light and electron microscopy. The bundles may travel from limbus to limbus where they continue as scleral fibres, and/or

they may end in the subepithelial layers. Some circular and oblique fibres were also found near the limbus. Similar findings were obtained in corneas of other mammals.

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ELECTRON MICROSCOPY OF THE HUMAN CHOROID

I. CELLS AND SUPPORTING STRUCTURES

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INTRODUCTION

The cytology of the choroid has been studied by light microscopy and other methods for many years. Most cells which have been classified as a result of these studies are well-known cell types. Little is known of the fine structure of the choroidal cells, and there are no electron microscopic reports of their morphology. Electron microscopic studies reveal something of the detailed structure of the melanocytes and other cells of the choroid and in part explains their function.

This initial study presents the principal cell types which have been encountered by us in four human eyes.

METHODS AND MATERIALS

The choroidal tissues studies were obtained from four human eyes enucleated for malignant melanoma of the choroid. The tumor in one eye involved the ciliary body and anterior choroid of a man 58 years of age. The other three tumors were in the choroid alone, and the patients' ages were 65, 65, and 70 years. The normal choroid on the side opposite the tumor was used for these studies.

In each instance, as soon as the enucleation was completed, a coronal section, through the eye, was made behind the lens and the anterior structures removed. The posterior portion of the eye was bisected meridionally in the appropriate plane. The

portion containing the tumor was fixed in formalin. The remainder of the tissue was immersed in one per cent osmic acid (4°C) buffered to pH 7.4 with barbital buffer. During immersion in the fixative the retina was gently stripped from the choroid. The attachment of the choroid to the sclera was loosened to allow fixative to penetrate the choroid and retina from the outer side. All this was accomplished within two to three minutes after enucleation. Using the optic disk as the center, five semicircular strips of choroid and retina were formed, the last including the ora serrata. These strips were cut into one to two millimeter squares and placed in fresh cold osmium fixative for an additional two-hour period.

The tissues, then, were rinsed briefly in buffer, dehydrated in a graded series of alcohols, infiltrated overnight in the refrigerator in a 4:1 mixture of n-butyl methacrylate and methyl methacrylate, and finally were embedded in this plastic mixture containing one per cent Luperco CDB as a catalyst. The plastic was polymerized with ultraviolet light.

Sections were cut on the Porter-Blum ultramicrotome at 1 μ thickness and stained routinely with toluidine blue for study with the light and phase microscope. Thin sections were cut with a microtome setting of 1/40 μ and were selected for thinness on the basis of interference colors. Sections were mounted on formvar-coated copper grids. Alternate grids were treated with uranium acetate and some were stabilized with a thin layer of carbon in the vacuum evaporator. They were examined in an RCA EMU 3E electron microscope fitted with a 35 to 40 μ objective aperture. Photographs were made at 5,000 to 16,000 original magnification and enlarged as desired.

* From the Department of Ophthalmology and the Francis I. Proctor Foundation for Research in Ophthalmology, University of California School of Medicine. This investigation was supported by Public Health Service research grants B-1229 and B-1782 from the Institute of Neurological Diseases and Blindness, National Institutes of Health. Read at the Midwinter National Meeting of the Association for Research in Ophthalmology, New Orleans, Dec. 7, 1960.

OBSERVATIONS

Supporting Structure. The choroidal supporting tissue fills the spaces between and among the blood vessels, nerves, and cells. It consists of a homogeneous ground substance in which collagen fibers are found. These fibers are single or arranged in small bundles. The usual 640 Å periodicity is observed. The collagen is more organized and abundant around the arterial vessels than around veins and capillaries. The direction of the fiber is mainly parallel to the long axis of the vessels, whereas in the interspaces the fibers course in many directions. Collagen fibers become less numerous toward the supra choroidea. Finally in the outer choroid only elastic fibers are apparent among the lamellae. Collagen fibers occasionally are found in the suprachoroidea but only in relation to nerve bundles.

Large numbers of elastic fibers are found throughout the choroidal stroma at all levels. They also are numerous in the adventitia of the vessels. The fibers are flat and ribbon-like in some areas, measuring up to 13 μ in length, and in others they appear as circular cross sections measuring about 0.3 μ in diameter (Fig. 1). The fibers are moderately electron dense, homogeneous, and have no membrane or condensation at the surface.

CELLS

a) *Suprachoroidal layer* (Fig. 2). The cells of the suprachoroidea either are pigmented or nonpigmented.

The principal *pigmented* cell of the suprachoroidea is the flat, branched melanocyte whose cytoplasm is filled with dense brown pigment granules. With the electron microscope these granules measure 0.3 to 0.4 μ in diameter. Some are homogeneous and uniformly dense while others are composed of aggregates of smaller granules varying in size from 20 to 80 m μ . These cells have a flattened nucleus surrounded by a double membrane, a well developed Golgi apparatus, and a few mitochondria, which measure 7 μ in length by 0.1 μ in diameter. There is a relative paucity of the vesicular and lamellar

elements of the endoplasmic reticulum. The cell processes are extremely long and fine and are difficult to follow in thin sections. The ultimate extensions of the cell branches contain little or no pigment. Macrophages containing pigment also are seen in this area. They will be described later.

The *nonpigmented* cell of the suprachoroidea is about the same size as the pigmented one, but seems to have a larger nucleus (Fig. 2). The cytoplasm contains few vesicular and almost no lamellar elements of endoplasmic reticulum. There are numerous free dense granules, possibly representing RNA-rich granules. The cytoplasm frequently contains a few homogeneous granules measuring 0.3 μ . These cells are bipolar and resemble fibroblasts in form but not in cytoplasmic content.

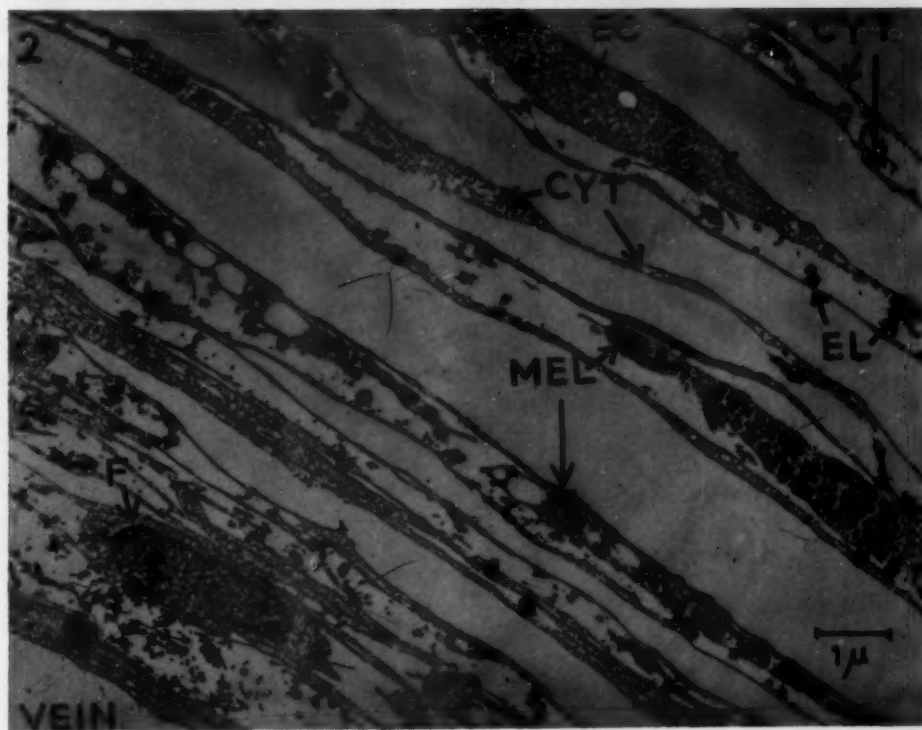
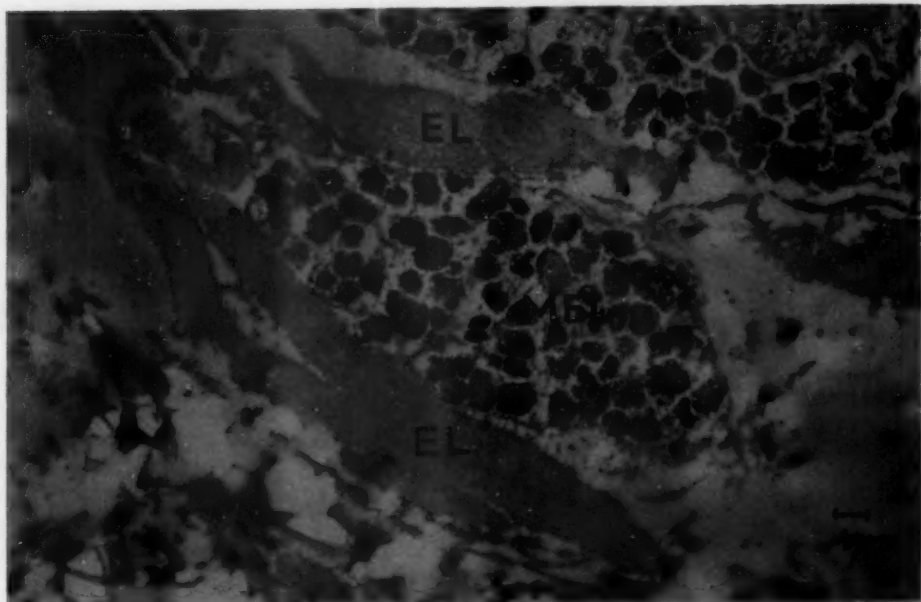
b) *The Choroid.* The choroid also contains both pigmented and nonpigmented cells. Among the *nonpigmented cells*, Schwann cells, the ganglion cells and neurons constitute the greatest number. These will be described in another paper.

Three types of *pigmented* cells can be seen in 1 μ thick sections: 1) large branching melanocytes filled with brown or golden-brown pigment of fairly uniform size; 2) round or oval cells with a pale, uniform cytoplasm which exhibits little or no branching, and have a fine sprinkling of pigment; 3) large cells containing irregular masses of



Fig. 1 (Feeney and Hogan I). Electron micrograph of elastic fibers in the stroma of the choroid. The fibers or ribbons (EL) are cut semilongitudinally. An arm of the cytoplasm of a melanocyte (MEL) filled with melanin pigment is seen between the elastic fibers. $\times 11,000$

Fig. 2 (Feeney and Hogan I). Electron micrograph of the suprachoroidal lamellae and part of a vein in the outer choroid. Strips of pigmented cytoplasm are extensions of melanocytes (MEL). The nucleus and unpigmented cytoplasm of an "endothelial" cell is seen at EC. Thin strips of cytoplasm (CYT) extend through the suprachoroidal space and are probably extensions of the unpigmented "endothelial" cells. Elastic fibers (EL). Fibrocyte (F). $\times 5,000$



pigment and refractile substance and readily identified as macrophages.

Melanocytes. There is a continuous gradation between types 1 and 2, and it is easy to differentiate the typical types from each other. The branched melanocyte shows a majority of the melanin particles in the cytoplasm to be large (0.3μ) and of uniform density (Fig. 3). These large granules vary from round to oval to ellipsoidal when thin sectioned. The edges of the particle frequently are irregularly scalloped and less dense than the center. That these granules have a substructure is best determined by examination of their edges, where microgranules measuring $30 m\mu$ can be seen. Most melanocytes have fewer compact melanin granules which show considerable variation in their size, shape, and consistency. Some of these are loose aggregates of small dense particles combined to produce a melanin granule less than 0.3μ in size. Others show an aggregation of the particles into strands, producing the effect of striations.

The amount of cytoplasm depends on the number of melanin granules; in some cells 70 percent of the total area may be occupied by granules. Mitochondria are scarce. The Golgi apparatus is well developed and does not appear to contain pigment. The rough-surfaced endoplasmic reticulum is largely vesicular in form and there are many dense free granules, presumably of RNP. Numerous smooth-surfaced endoplasmic reticulum vesicles are found throughout the cytoplasm. Pinocytotic vesicles measuring 60 to $80 m\mu$ in diameter are found in most cells; they appear only in a localized area at or near the plasma membrane. Microvillous projections of the cytoplasm are seen frequently.

The type 2 cell appears in a number of forms; one is lightly pigmented and the cytoplasm has an exceptional number of mitochondria (Fig. 4). The scattered pigment particles measure less than 0.2μ (just below the limits of the light microscope) and are composed of microgranules measuring approximately $20 m\mu$. They are found within

smooth-surfaced, membrane-bounded vesicles.

The mitochondria generally are cylindrical and measure approximately 1μ in length and 0.3μ in width. However, filamentous forms, 4μ long, have been seen. The internal double lamellae are well developed. The endoplasmic reticulum is not abundant, and is vesicular. Many free granules are scattered in the cytoplasm. There are numerous smooth-surfaced vesicles and many bodies containing multiple vesicles. The oval membrane, and occasionally pores are found in this membrane. Microvillous projections of the superficial cytoplasm and pinocytotic vesicles usually are found. A layer of material resembling a basement membrane may be seen along portions of the plasma membrane, but it does not appear to surround the cell completely.

Another of these cells is characterized by numerous striated melanin granules, and there is an axial arrangement of the melanin particles within the granule (Figs. 5, 6). This axial arrangement seems to be produced by dense pigmented strands loosely arranged to produce an ellipsoidal, banded, melanin granule with the strands converging at the poles of the ellipsoid. The individual striated melanin granules frequently are found in clusters of a dozen or more, but the clusters are not surrounded by a membrane as are those of the macrophage. The strands in



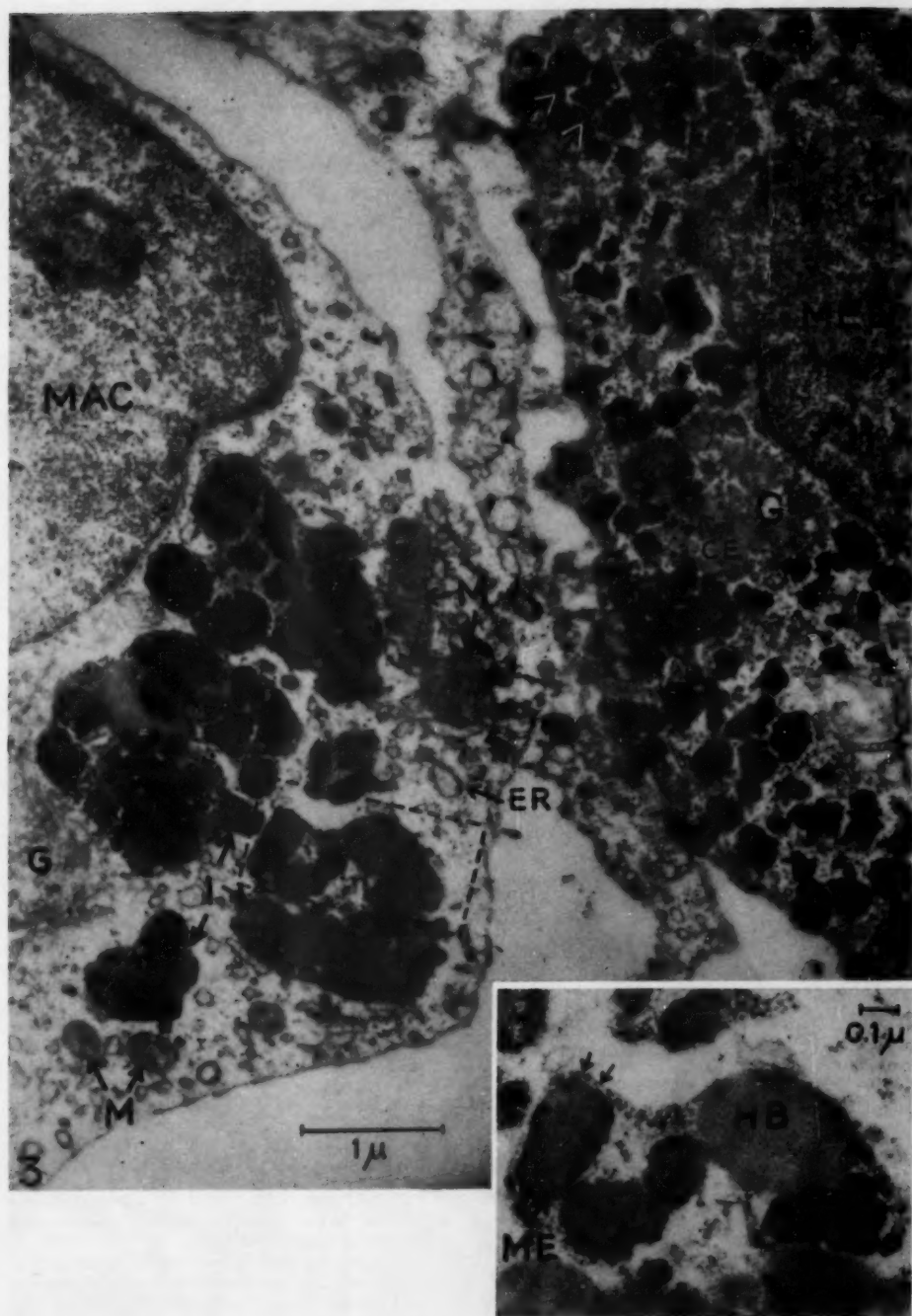
Fig. 3 (Feeney and Hogan I). Electron micrograph comparing the characteristics of a melanocyte (MEL) and a macrophage (MAC). The melanin particles of the melanocyte are dispersed throughout the cytoplasm and the granules are of fairly uniform size. Two striated granules are seen at the arrows. The Golgi region (G) of the cytoplasm contains portions of the centrioles (CE).

The pigment of the macrophage is seen as inclusions only in vacuoles bounded by membranes (I). The cytoplasm contains mitochondria (M), a Golgi apparatus (G), and endoplasmic reticulum (ER).

$\times 22,000$

(Insert) Enlargement of the outlined area of the macrophage. Melanin microgranules (arrows) are seen in the membrane-bounded vacuole. A homogeneous dielectronic body (HB) and a dense homogeneous melanin granule (ME) also are seen in the inclusion.

$\times 50,000$



occasional granules in the same cell may be cut at right angles to the plane of the strands, producing a picture of clumps of microgranules. These cells also contain numerous mitochondria but the number is fewer than in the previously described type.

Still others of these cells resemble the adult melanocyte in their paucity of mitochondria and more densely packed pigment (Figs. 7, 8).

The type 3 pigmented cell is the choroidal macrophage (Fig. 3). They are seen frequently and are characterized by large inclusions in the cytoplasm measuring 1 to 3 μ in diameter, although larger and smaller inclusions occur. The inclusions contain a variety of ingested material. There are dense round or oval granules measuring about 0.3 μ and these are indistinguishable from mature melanin granules. Other smaller dense granules are cylindrical and vary in size from 0.04 μ to 0.1 μ and resemble the subparticles of the melanin granule. Very fine particles of almost uniform size are found throughout the inclusion and frequently are lined up along the limiting membrane of the inclusion vacuole. These fine particles measure 20 m μ in diameter. Moderately dielectronic homogeneous bodies measuring 1 μ long by 0.3 μ in width are common in the inclusion vacuole. These are oval or kidney shaped with poorly defined margins.

The macrophage contains numerous elements of rough-surfaced endoplasmic reticulum which mostly are vesicular in form. There are many granules, possibly of RNP, arranged singly and in clusters. The Golgi complex is well developed. Numerous smooth-surfaced vesicles are found throughout the cytoplasm. The mitochondria are large, measuring 1 μ in length and 0.3 μ in width with well developed cristae.

A number of nonpigmented cells are found in the stroma. Those connected with blood vessels and nerves will be described separately.

Fibrocytes. The choroidal fibrocyte is identifiable as a cell which most often is without pigment and contains a distinct

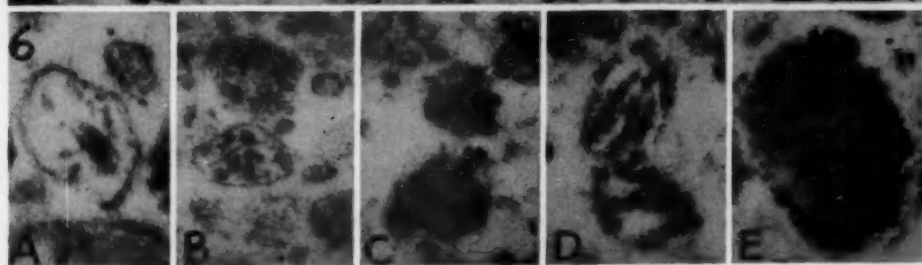
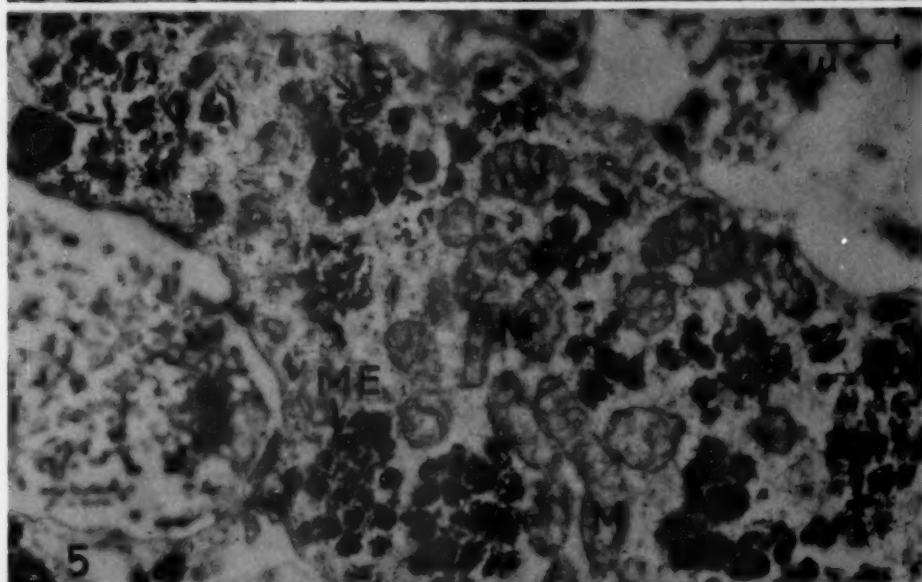
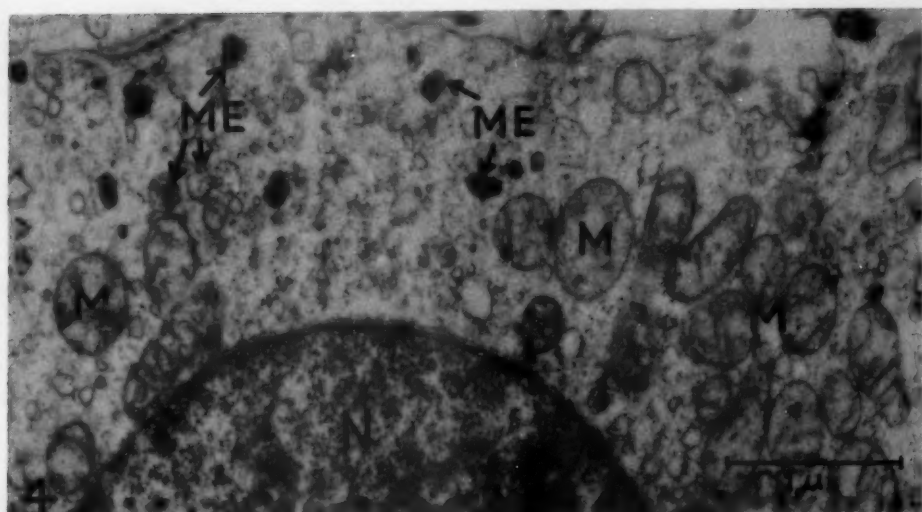
lamellar endoplasmic reticulum (Fig. 9). Because of its multiple branches the cell is never seen in its entire length in thin sections. Portions of its cytoplasmic processes usually can be recognized throughout the choroid. The nucleus measures about 8 μ and contains evenly dispersed fine particles. The nucleoli are seen to be composed of a denser aggregation of particles. The double nuclear membrane has pores. The cytoplasm contains lamellar rough-surfaced endoplasmic reticulum. The lamellae of the endoplasmic reticulum frequently lie in 2 or 3 parallel rows near the nucleus. Other parallel rows extend into the cell processes. Small vacuoles and vesicles dotted with granules (vesicular endoplasmic reticulum) are commonly found. Smooth-surfaced vacuoles and vesicles are numerous. The mitochondria are small (1 μ long by 0.2 μ wide) and are not numerous as compared with the type 2 pigmented cell. The Golgi complex is an ill-defined area of smooth-surfaced vacuoles and vesicles. The cytoplasm usually contains a few medium-dense homogeneous bodies surrounded by a membrane. These oval bodies vary in size from 0.2 μ to 0.7 μ in length and 0.15 μ to 0.3 μ in width.

Fig. 4 (Feeney and Hogan I). Electron micrograph of a portion of a small round melanocyte. Tiny melanin particles (ME) are seen within membrane-bounded vesicles. Some vesicles contain only one or two microgranules (arrows). Other vesicles contain many particles in a feathery aggregation. There are many mitochondria (M) in the cytoplasm. Nucleus, N. $\times 26,000$

Fig. 5 (Feeney and Hogan I). Electron micrograph of a portion of a melanocyte in which the melanin granules are larger, denser, and more numerous. Some of the granules appear to be solid. Others are striated. Two granules (arrows) show striations which are loosely arranged in an ellipsoid. These are enlarged in Figure 6D. There are numerous mitochondria (M) in the cytoplasm. $\times 26,000$

Fig. 6 (Feeney and Hogan I). Electron micrographs of various forms of melanin granules seen within melanocytes. (See text).

- A. Vacuole containing two beaded filaments.
- B. Vacuole containing heavily beaded filaments.
- C. Vacuoles shown in cross-section to show profiles of filaments.
- D. Ellipsoidal, banded, melanin granule.
- E. Large, dense granule. $\times 70,000$



Lymphocytes occasionally are seen just outside the walls of the veins and capillaries (Fig. 10). They measure $4\ \mu$ to $7\ \mu$ in diameter and have a large round or indented nucleus surrounded by a double membrane and not much cytoplasm. The mitochondria measure about $0.4\ \mu$ in diameter. Dense bodies measuring $0.35\ \mu$ in diameter surrounded by a membrane are occasionally seen. Organized endoplasmic reticulum is scarce, but there are numerous free dense granules. Many pleomorphic cytoplasmic projections extend from the cell surface.

Mast Cells. Mast cells are beautifully demonstrated by light microscopy with the toluidine blue stain of the $1\ \mu$ thick sections. Two types of mast cells are identifiable in the electron microscope:

a) A large (10 to $12\ \mu$ in diameter) rounded cell packed with large granules of lighter density than those of the dense melanin granule (Fig. 11). The granules of this mast cell are oval and may be as large as $2\ \mu$ in length, however, the usual granule is between 0.5 to $1\ \mu$ in length and 0.3 to $0.6\ \mu$ in width. The granules are homogeneous except that they occasionally contain a small ($40\ m\mu$) dense body or dense-rimmed vesicles of about $0.1\ \mu$ diameter. Dense lipid inclusions occasionally are found in the cytoplasm. The mitochondria are small (about $0.7\ \mu$ in length by $0.15\ \mu$ in width) with well developed cristae and lie between and among the granules, often wedged between a granule and the plasma membrane. The endoplasmic reticulum consists largely of small vacuoles and short canaliculi.

b) A small ($9\ \mu$ in length by $4\ \mu$ in width) dense cell with an oval nucleus. The cytoplasm contains numerous clusters of ring-shaped double membranes (Fig. 12). The rings are cross-sections of hollow cylinders and are slightly oval and very uniform in size. They measure 85 to $90\ m\mu$ in length and 45 to $60\ m\mu$ in width. The double membranes forming the ring are 11 to $13\ m\mu$ apart. There may be 3 to 11 rings in a cluster. Some of the clusters are more dense and are surrounded by another double mem-

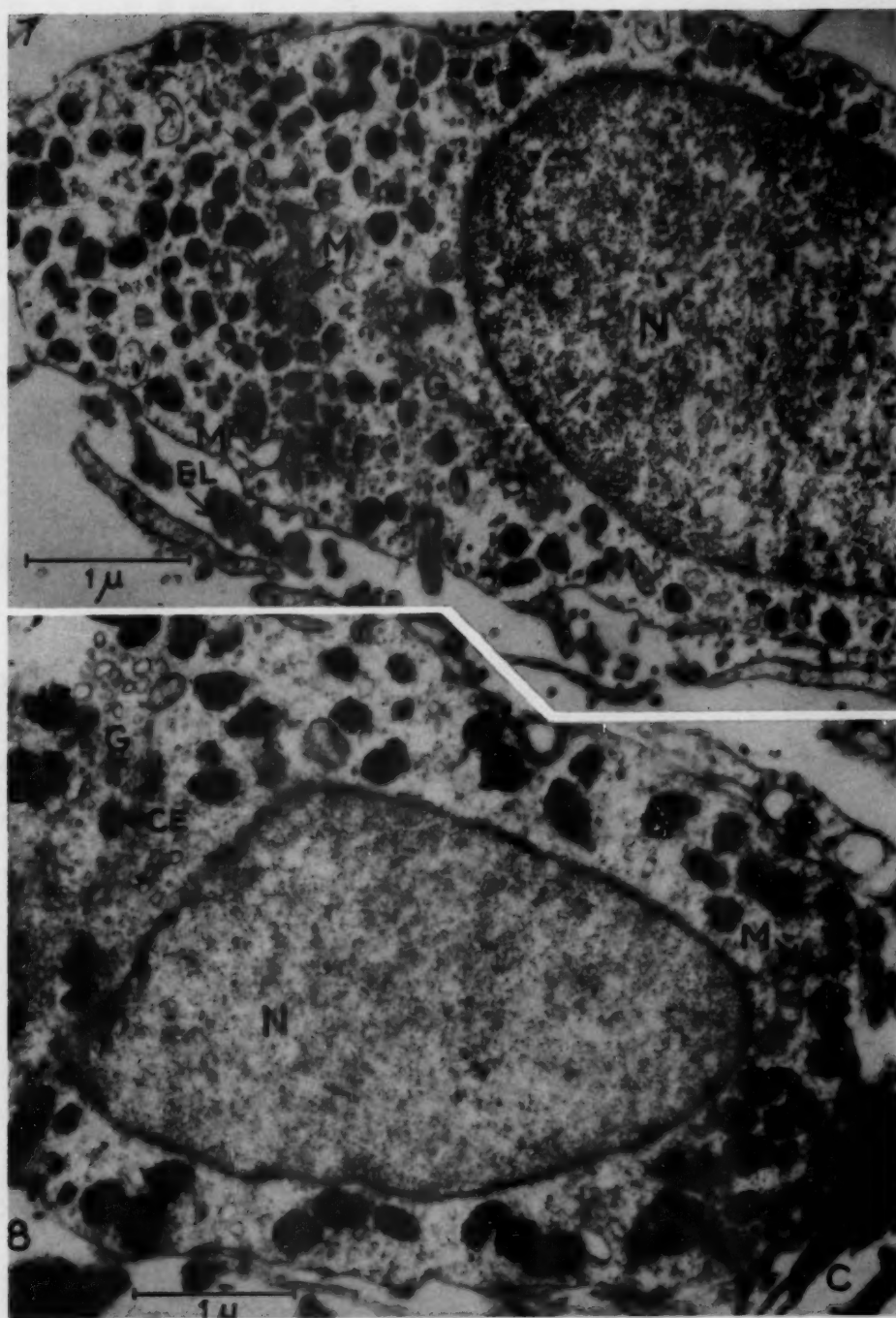
brane to form a body or granule 0.35 to $0.4\ \mu$ in diameter. Oblique sections through a cluster show the tubular nature of the rings.

The rest of the cytoplasm contains small mitochondria 0.3 to $0.35\ \mu$ in length and 0.12 to $0.14\ \mu$ in width. There are occasional lipid droplets. Organized endoplasmic reticulum seldom is seen although there are abundant free granules in the cytoplasm. The entire cell is filled with a feathery flocculent ground substance which gives a generalized electron density to the cell.

Unclassified cell. One additional cell has been seen in the choroid which is not classified although it may be a variation from one of the normal cell types (Fig. 13). This cell contains one or more lamellar membranous whorls. The whorl is composed of 10 to 14 layers of double membranes enclosing a core composed of vacuoles, dense irregular masses, and finely granular material. The double membranes are continuous with the smooth-surfaced endoplasmic reticulum of the cytoplasm. The innermost layer is continuous around the vacuoles at the core. The distance between membrane pairs is 13 to $14\ m\mu$ and the distance between successive layers of double membranes is about 14 to $16\ m\mu$. Paired membranes diverge in certain areas of the whorl to enclose portions of cytoplasm. The cytoplasm in other parts of the cell contains small mitochondria measuring $0.2\ \mu$ in diameter, rough-surfaced endoplasmic reticulum of the lamellar and

Fig. 7 (Feeney and Hogan I). Electron micrograph of a melanocyte. The melanin granules are surrounded by a membrane. There is a variation in the number or concentration of melanin microgranules in the vesicles. There are few mitochondria (M). The Golgi zone (G) is free of melanin particles. Nucleus (N). Elastic fibers (EL) are seen in the stroma. $\times 25,000$

Fig. 8 (Feeney and Hogan I). Electron micrograph of the nuclear region of a large melanocyte. A small portion of the nucleus (N) occupies the center of the figure. Parts of the Golgi apparatus (G) and the centrioles (CE) are seen near the nucleus. The melanin granules in the cytoplasm are large and of uniform density and size. Collagen fibers (C) are seen in the adjacent choroidal stroma. $\times 25,000$



vesicular types, clusters of melanin granules, and homogeneous dielectronic bodies. The rounded nucleus measures about 7 μ in diameter.

DISCUSSION

Melanocytes have been studied in a number of tissues including the human and other mammalian epidermis,^{1,2} human hair,³ mouse melanoma,^{4,5} human malignant melanoma,^{6,7} and developing retinal pigment epithelium.⁸

Selby¹ in a study of fetal and adult human skin found the fetal melanocyte to contain mitochondria, numerous tonofilaments, many free RNA-rich particulates, and only very occasional vesicles of endoplasmic reticulum. The adult human skin showed melanocytes which contained pigment granules having an irregular angular outline. They were extremely electron-dense. Their smallest diameter and length was significantly greater in the Negro. In this early study no mention was made of the internal structure of the melanin particle.

Birbeck et al.³ studied the development of the melanin granule in human hair and found some of the particles to be aggregated to form parallel lamellae, often forming a succession of cylindrical or ellipsoidal shells.

Dalton⁶ recently has presented evidence to show that melanin granules form in the Golgi apparatus of the melanoma cell. Wellings and Siegel^{6,7} studied human melanoma cells and also concluded that the Golgi apparatus was the site of melanin-formation.

Moyer⁸ studied the origin, development, and genetic variation of the melanin granules in the retinal pigment epithelium, iris and choroid of various inbred mice. He recognized four stages in the formation of the melanin: 1) an initial smooth-surfaced, double membrane-bound vesicle containing diffusely granular material; 2) the vesicle became filled with fibrous material; 3) the fibrous material became beaded with melanin precipitates, producing a densely striated granule; and 4) the strands containing the particles finally became compressed and a solid melanin granule formed in which no

particulate structure could be made out.

Our findings confirm those of other investigators regarding the structure of the melanin granule. The melanocytes of the choroid differ from those of the epidermis, hair, and malignant melanoma cells in two respects: 1) The melanin granules in choroidal melanocytes are larger, rounder and much more numerous; 2) a given cell usually shows the majority of the pigment granules to be at one level of development, i.e., cells containing scattered pigment in vesicles rarely contain solid granules at the same time. Conversely, cells containing solid or densely striated granules show few of the vesicles containing pigment microgranules.

The melanocytes of the choroid are easily recognized by their size, heavy branching, and large number of rather uniform dense melanin granules which fill the cytoplasm and extend into the processes.

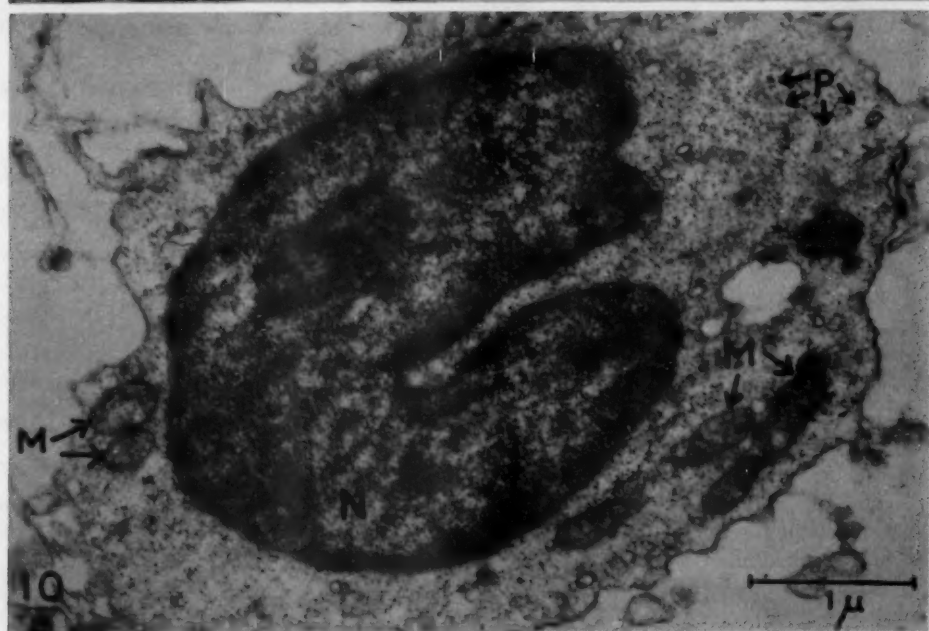
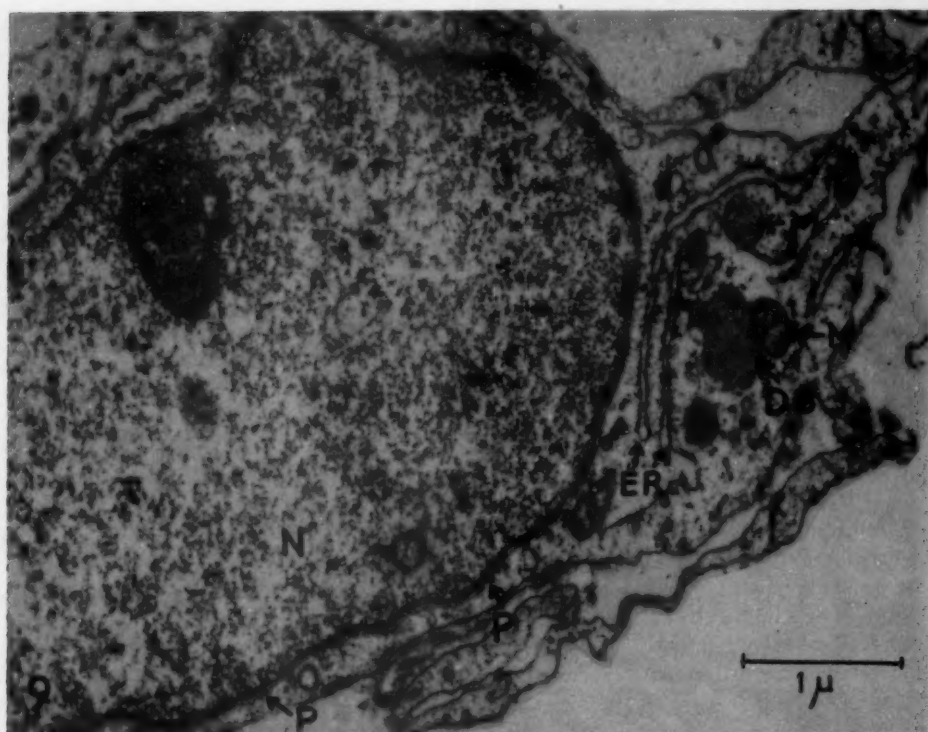
Other melanocytes are smaller, less heavily pigmented, and the particles have a striated or granular appearance.

Macrophages from a variety of tissues have been studied with the electron microscope.^{9,10,11,12} Choroidal macrophages differ from those in other tissues only in the content of their inclusions; the majority of the contents of each inclusion consists of melanin granules. Pigment generally can be distinguished from phagocytosed erythrocyte particles by the internal structure of the pig-

Fig. 9 (Feeney and Hogan I). Electron micrograph of a fibrocyte. The large nucleus (N), containing a nucleolus, is surrounded by a double membrane which occasionally is dotted with dense particles (P). The cytoplasm contains rough-surfaced endoplasmic reticulum (ER) which appears as double membranes with dense particles adherent to the outside surfaces. A homogeneous dense body (DB) and mitochondria (M) are seen in the cytoplasm. The cytoplasmic processes extend a considerable distance from the cell and are not included in this micrograph. $\times 25,000$

Fig. 10 (Feeney and Hogan I). Electron micrograph of a lymphocyte seen in the stroma of the choroid near a venule. The nucleus (N) characteristically indented is seen. The cytoplasm is relatively dense and contains several mitochondria (M).

Ribonucleoprotein particles (P). $\times 25,000$



ment granule. The cytoplasmic inclusions containing melanin granules are isolated from the cytoplasm by a membrane which helps distinguish the macrophage from a melanocyte.

Hibbs et al.¹³ recently described cells, assumed to be mast cells, from biopsies of human digital and abdominal skin and subcutis, and suction biopsies of human gastric mucosa. They classified these cells into two groups: a) a spindle-shaped cell with large homogeneous granules, and b) an oval or round cell whose granules were composed of "lamellar scrolls." The mast cells found by us in the choroid are nearly identical with those described above. Our classification of mast cells, therefore, is based on their work. Their elongated spindle cell was "chromaffin positive" (a reaction common to cells of the adrenal cortex and believed to be due to epinephrine or norepinephrine or both). The oval cell has all the features of the classical mast cell.

Cells containing lamellar whorls (myelin figures), similar to those found by us, have been described in the pigment epithelium of the retina,^{14,15} reticuloendothelial cells containing phagocytosed erythrocytes,¹² testicular cells,¹⁶ muscle cells,¹⁷ and leukocytes in the Chédiak-Higashi syndrome.¹⁸ These figures have been produced in vitro from hydration of phospholipids.^{19,20} The presence of pigment inclusions within this choroidal cell suggests that it is a macrophage, however, further studies are necessary before a classification can be made.

Smooth muscle cells were found only in the muscularis of the arteries and not in the stroma of the choroid posterior to the equator.

To us the "endothelial cells" of the suprachoroida are those described as having large nuclei and the usual cytoplasmic organelles. They differ in many respects from endothelial cells of blood vessels, so there seems to be no justification for considering them as endothelium. They do not line a cavity, nor cover a trabecular structure. These cells, along with the elastic fibers (which they do

not cover, but merely touch occasionally as they course their separate ways), and melanocytes form the suprachoroidal lamellae.

The elastic fibers of the choroidal stroma and the suprachoroida are not different from those found in arteries, lung, or the trachea.^{21,22,23} The great numbers of elastic fibers present were an unexpected finding, although a study of the 1 μ thick sections with the phase microscope indicated that more elastic tissue was present than previous studies would indicate. The small dimensions of many of the fibers render them invisible to the light microscope.

CONCLUSIONS

1. The melanocytes of the choroid can be classified into two groups:

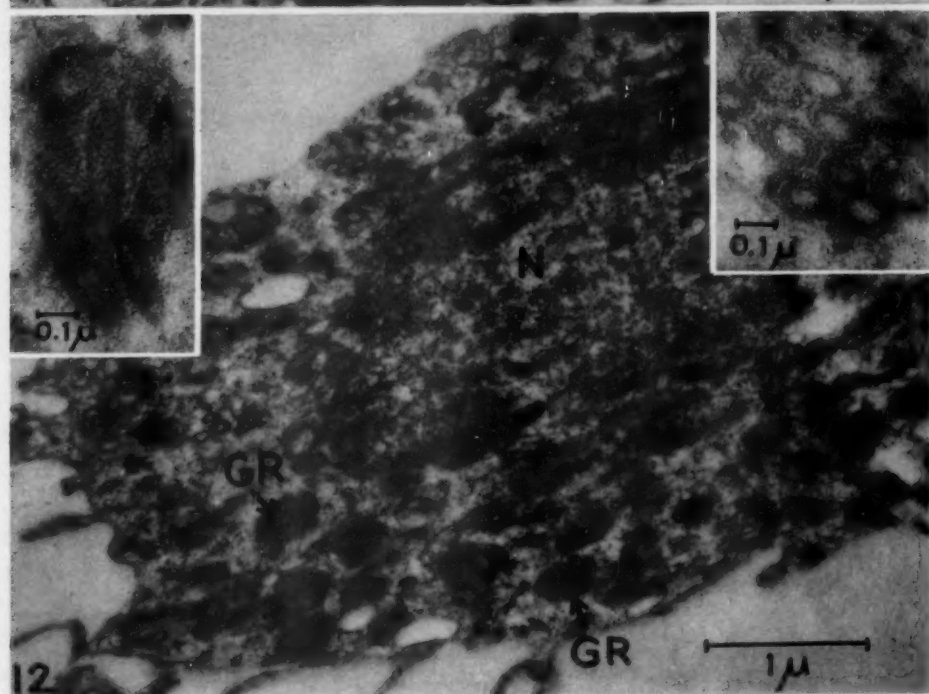
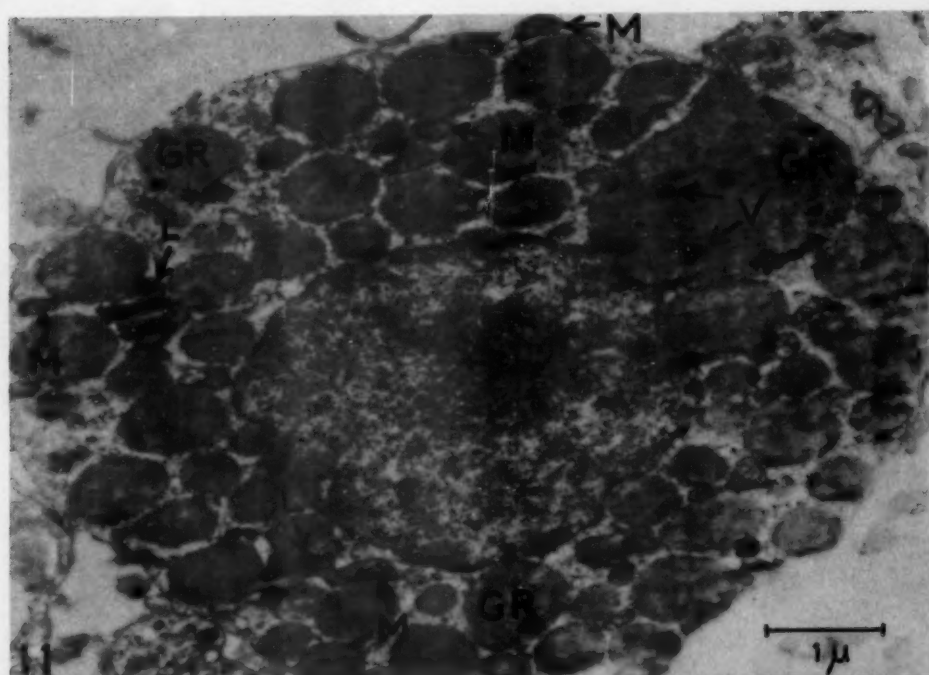
a) *The large branched melanocyte.* The cytoplasm is filled with many large, dense melanin granules of uniform size, and the granules extend out into the branching to their terminations.

b) *A smaller oval cell with less branching.* The cytoplasm contains numerous mitochondria and tiny pigment particles. The particles have an internal structure and therefore differ from those of the "type a" cell. The structure of the particles varies from a vesicle containing powdery granules to one containing lamellae beaded with pigment particles. Cells of this type containing

Fig. 11 (Feeny and Hogan I). Electron micrograph of a *large* mast cell. The cytoplasm contains large homogeneous granules (GR), some of which contain dark rimmed vesicles (V). Lipid droplets (L) occur between the granules. Small mitochondria (M) are seen among the granules, and near the plasma membrane. Note that the magnification of this cell is two-thirds that of the other cells in this paper. $\times 18,000$

Fig. 12 (Feeny and Hogan I). Electron micrograph of a *small* mast cell. The granules (GR) in the cytoplasm vary in size, shape and density. $\times 25,000$

The two insets show the appearance of these granules at higher magnification. On cross-section (right) the granules are seen to be composed of clusters of double membrane-bounded rings. On longitudinal section (left) they appear as tubes or cylinders associated with a homogeneous material. $\times 70,000$



vesicles and powdery granules never show large solid melanin granules. Cells containing a few vesicles, and many striated melanin granules, often show occasional solid melanin granules. Those cells mostly containing large numbers of solid melanin granules occasionally show a few striated granules.

2. The choroidal macrophage contains inclusions of phagocytosed pigment granules and other material within membrane-bound vacuoles.

3. Mast cells frequently are seen in all choroidal layers. Two types have been found: one in which the granules are large and homogeneous; the other in which the granules are composed of double membrane-bound tubules.

4. Fibrocytes are numerous and are recognized by their long cytoplasmic projections and the typical appearance of their endoplasmic reticulum.

5. Smooth muscle cells are found only in the muscular layer of the arteries.

Fig. 13 (Feeney and Hogan I). Electron micrograph of a cell (macrophage?) containing two myelin figures. They are composed of whorls of smooth-surfaced double membranes. About 15 pairs of membranes comprise the walls of the figure. The center of each figure contains vacuoles (V), dense material resembling melanin (ME), and membranous bodies (MB). The cytoplasm of the cell also contains inclusions of melanin (ME) and homogeneous bodies (HB). X18,000

(Inset) Lamellae of the myelin figure seen at higher magnification. The membrane pairs divide and surround the vacuoles (V). X34,000

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6. No evidence is found for classifying the large unpigmented cells of the suprachoroida as "endothelial cells."

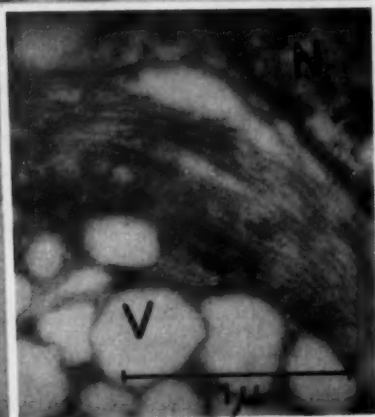
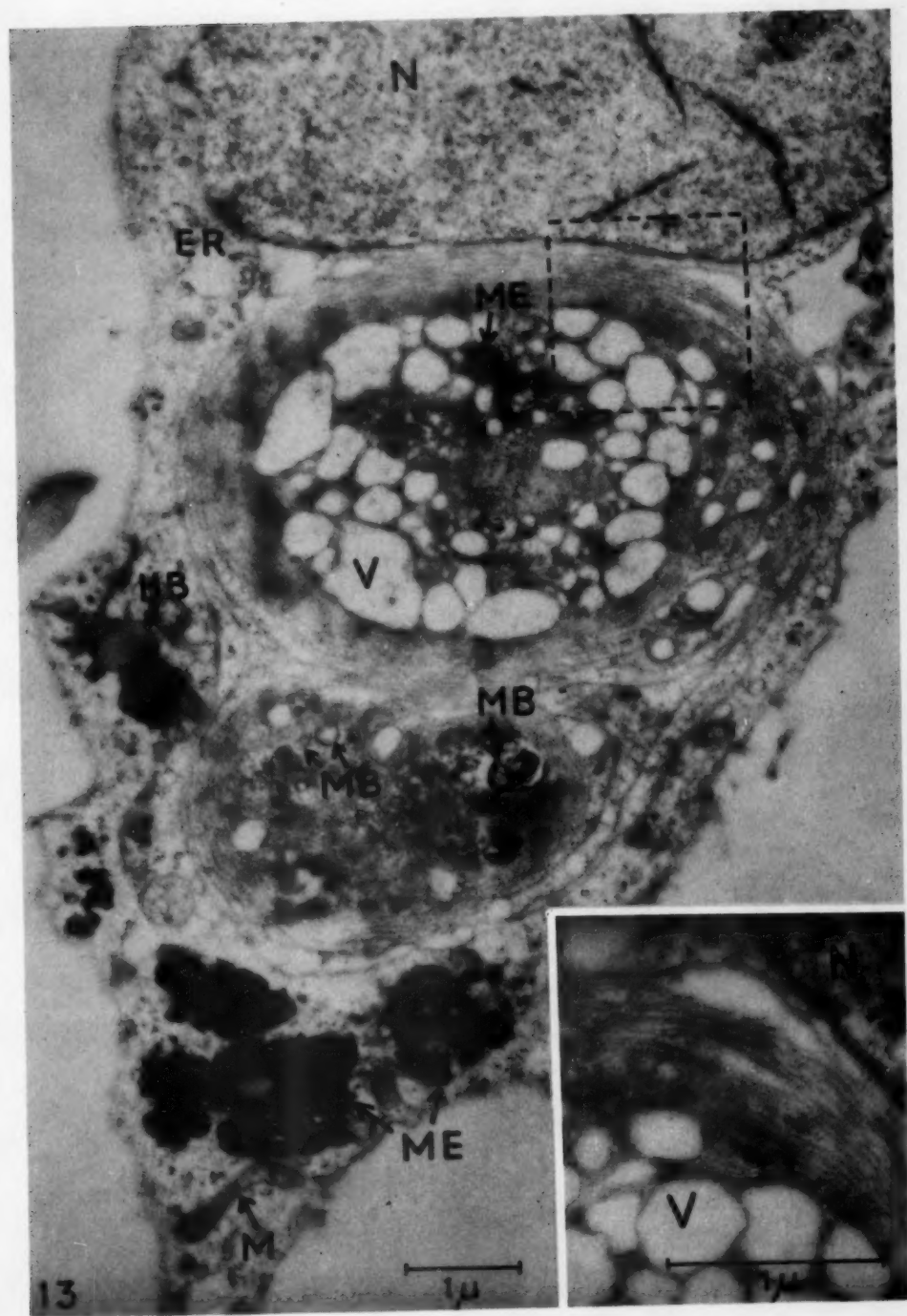
7. A cell of unknown type is reported, which contains a myelin figure.

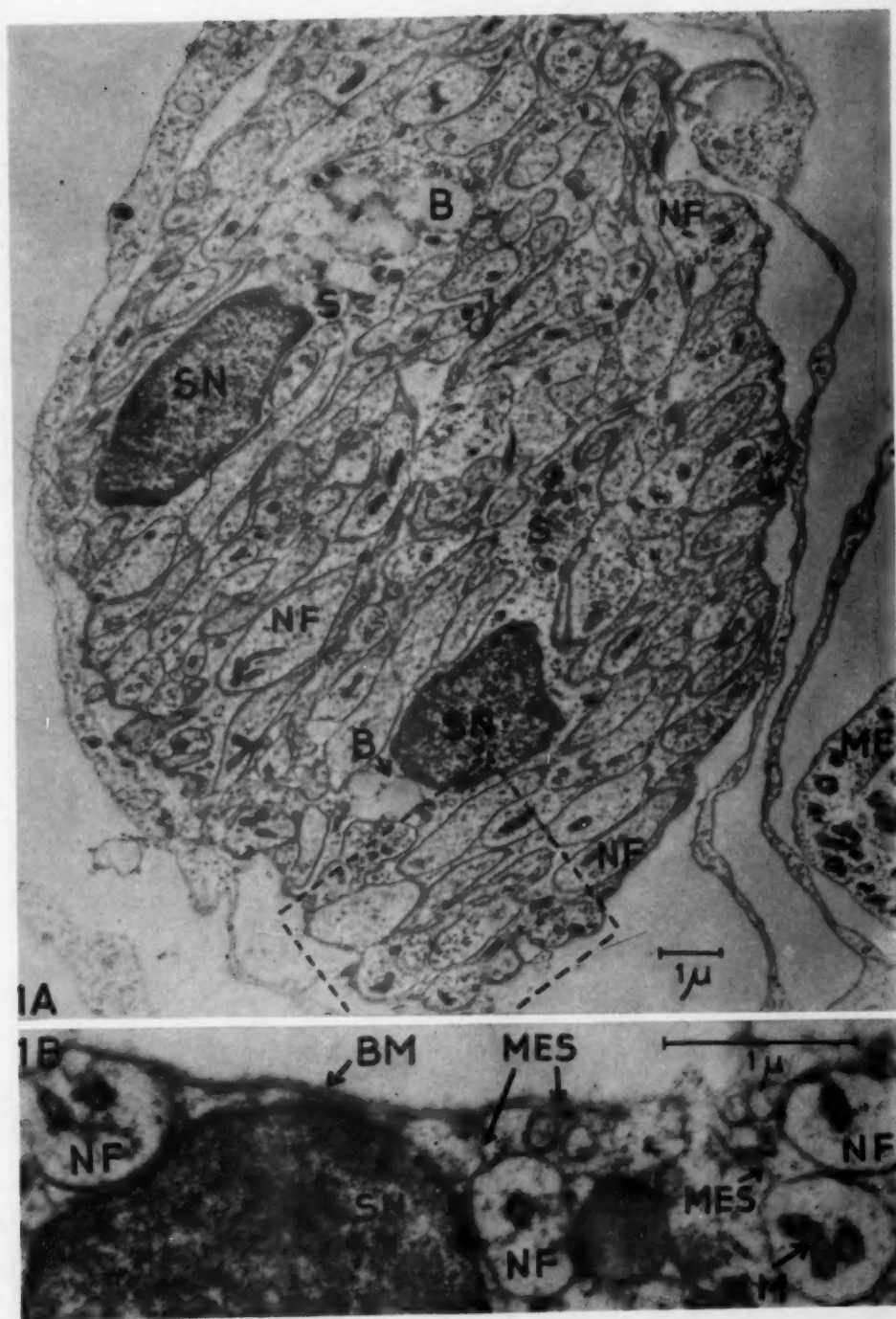
8. Large numbers of elastic fibers are found in all layers of the choroid. They are similar to those found in lung, trachea and arteries.

San Francisco (22).

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branches are multipolar ganglion cells. The nerve endings from these cells are in the choroidal blood vessels. Bietti² in 1899 found a very fine network of nerves throughout the layers of the choroid, especially around the arteries, and he observed club-shaped or special endings in the vessel musculature. He also found a nerve layer beneath the lamina vitrea.

According to Duke-Elder,³ the choroid is well-provided with branches of the ciliary nerves. These traverse the perichoroidal space, and break up into plexuses of increasing delicacy in the vascular layers and stroma of the choroid where their finest branches end by small spherical swellings in the musculature of the vessels. Associated with these plexuses are numerous multipolar ganglionated cells of the sympathetic type, the function of which is probably vasomotor.

Wolter⁴ employed the del Rio Hortega stain on normal human choroid and found it to have a very rich nerve supply. He showed two closely related and interconnected types of nerve fiber: a) ciliary nerve branches in the intervacular spaces; b) perivascular nerve fibers. He found the branches of the ciliary nerves to divide dichotomously, and to accompany blood vessels as parallel bundles. Branches of these bundles entered the vessel wall to ramify among the cells. No end plates were demonstrated. He described terminal nerve networks on the outer surface of Bruch's membrane in this eye, as well as nerves surrounding the capillaries of the choriocapillaris. This will be commented on in a subsequent paper.

METHODS AND MATERIALS

The methods and materials used in this study are identical to those described in a preceding paper.⁵

RESULTS

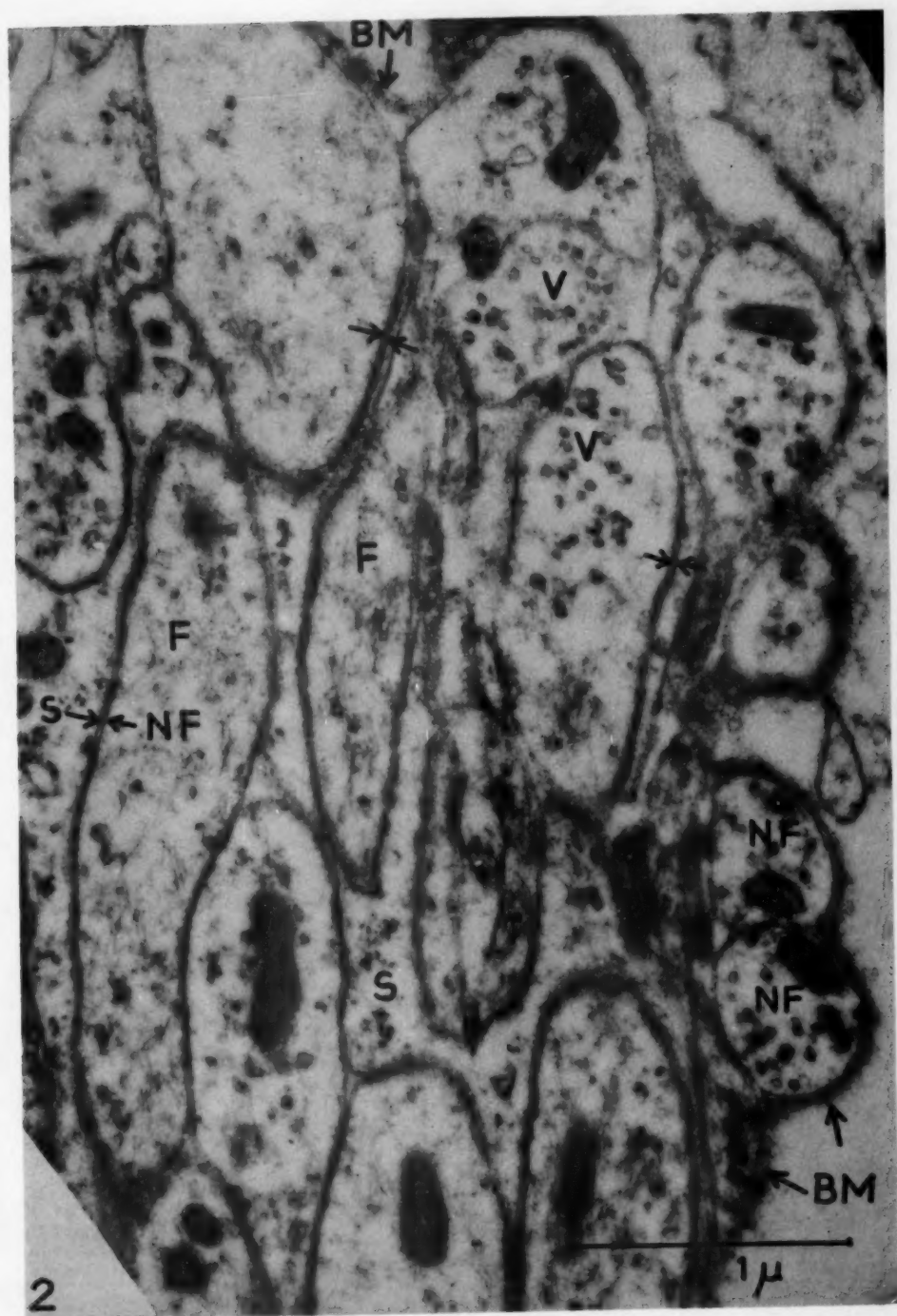
Plexuses, bundles, and single fibers are found throughout the stroma of the choroid except in the capillary layer.

The nerve bundles form large plexuses with nodal points which usually are marked by a large number of Schwann cell nuclei and several melanocytes. Observation of 1 μ sections with the light microscope shows these nodal areas to resemble a large multinucleated cell. The Schwann cell nuclei generally are found in the center of these bundles. The bundles in the suprachoroid (fig. 1A, B) contain up to 150 fibers, while those in the choroidal stroma have up to 50 fibers. Smaller bundles in the inner choroid may have only two or three nerves as they approach a blood vessel. The individual nerve fibers of the bundles are 0.5 to 1.5 μ in diameter. The plexuses are most prominent between the large vessel layers and are particularly numerous around the arteries. All of the nerve fibers we have encountered are unmyelinated, i.e., they have a single wrapping of Schwann cell cytoplasm. The only myelinated nerves in the choroid are those which lie in the suprachoroid.

The axoplasm of the nerve fibers contains many fine neurofibrils which are approximately 5 m μ wide (fig. 2). The mitochondria are relatively small. Vesicular endoplasmic reticulum may be seen in the axoplasm of the larger nerve fibers but rarely is present in the finer ramifications. Small vesicles 23 to 30 m μ in diameter, the so-

Fig. 2 (Feeny and Hogan, II). Electron micrograph of the periphery of the nerve bundle shown in Figure 1A. Nerve fibers cut obliquely show numerous neurofibrillae (F) in the axoplasm. Other nerve fibers contain numerous vesicles (V) which indicates they are sectioned near a synapse. Two small nerve fibers (NF) are not enclosed by cytoplasm of the Schwann cell; however, the basement membrane (BM) is continuous around them.

Arrows indicate profiles of two membranes: One is the plasma membrane of the nerve fiber (NF) and the other is the invaginated plasma membrane of the Schwann cell (S). $\times 45,000$



called synaptic vesicles, may be present in large numbers, presumably because of the proximity of the section to a synapse (fig. 3A). Other vesicles more than twice the size (65 to 90 m μ) of the synaptic vesicles also are found in the axoplasm of certain nerves. These larger vesicles appear to contain a homogeneous material and some fine particles (fig. 3B).

Almost all the nerve fibers have a neurilemma, formed by the Schwann cell. Most often the nerve fibers are found embedded in pockets and indentations of the Schwann cell. The external surface of the Schwann cell plasma membrane faces the axon plasma membrane regardless of how deeply the nerve fiber is embedded. The pocket enclosing the axon can be traced to the surface of the Schwann cell in favorable sections. The relationship of the axons to the Schwann cells can best be exemplified by comparison with the bowel and mesentery. The mesentery is composed of two leaves which enclose the bowel and anchor it to the abdominal wall. The Schwann cell membrane forms the mesentery (mesaxon) which encloses the axon. The two limits of the Schwann cell wall form the mesentery and connect with the surface of the cell (fig. 4). It is difficult to trace the mesaxon of a nerve which is embedded deep within the Schwann cell. Such axons are surrounded by two osmiophilic lines or membranes: a) the inner line is the plasma membrane of the axon; b) the outer one is the plasma membrane of the Schwann cell. The axons thus are extracellular to the Schwann cell, although they are embedded within them.

Frequently small axons are only partially embedded in the Schwann cell. In such instances one-half to two-thirds of the nerve is devoid of a neurilemma, but the exposed portion is covered by a basement membrane, continuous with that of the Schwann cell (fig. 2).

A nerve bundle which is sectioned through the nuclear region of its Schwann sheath generally shows the larger axons embedded

deeper in the cytoplasm than the small ones.

The Schwann cell nucleus is relatively small, measuring less than 5 μ (fig. 5). The cytoplasm contains a few small mitochondria, short segments of lamellar endoplasmic reticulum, clusters of free granules, presumably RNA-rich granules, and dense bodies about 0.2 μ in width. Frequently there are larger dielectronic homogeneous bodies measuring 1 μ or more in width. Melanin granules rarely are seen in the cytoplasm of the Schwann cell.

Numerous nerve fibers are found around the blood vessels, particularly the arteries and arterioles. Small bundles of nerves measuring 1 μ or less in diameter and containing 3 or 4 axons consistently are found in the adventitia of the arteries. Larger nerve bundles also are frequently seen in close proximity to blood vessels. Despite the apparent connection of nerves to vessel walls, actual contacts of nerve fibers with muscle cells have not been demonstrated. Endings resembling synapses have been found occasionally on the basement membrane surrounding the muscle cell wall (fig. 6). In these instances the axon widens

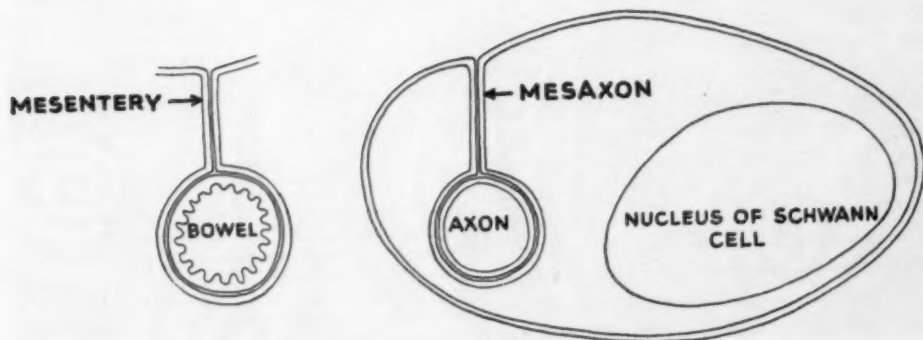
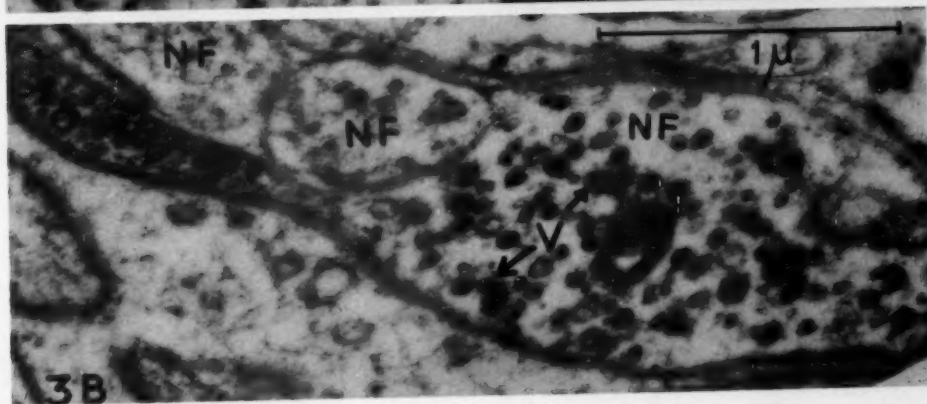
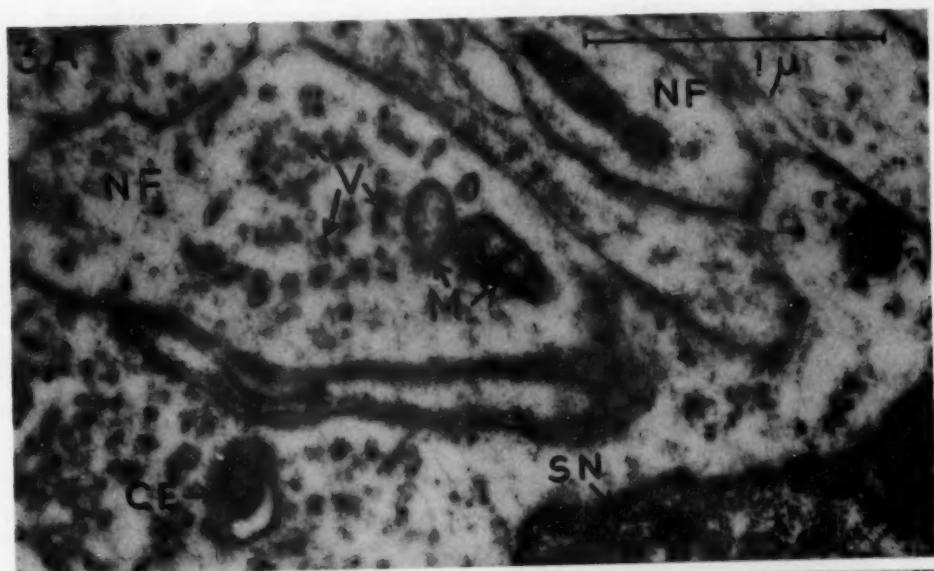


Fig. 3 (Feeny and Hogan, II). Electron micrograph comparing the two types of vesicles seen in nerve endings.

A. A nerve fiber (NF) contains small vesicles (V) typical of those found at synapses and neuromuscular junctions. A portion of the Schwann cell nucleus (SN) is visible and part of a centriole is visible (CE). The profiles of the nine peripheral filaments can be seen. Mitochondria (M) are seen in the axoplasm of the nerve fibers. $\times 45,000$

B. A nerve fiber containing dense vesicles (V) about twice the size of those in Figure 3A. $\times 45,000$

Fig. 4 (Feeny and Hogan, II). Diagrammatic analogy of the mesentery and mesaxon. The axon is embedded in the Schwann cell and is connected to the surface by a stalk, the mesaxon, analogous to the mesentery connection of the bowel to the abdominal wall.



abruptly to 1 to 2 μ and the Schwann sheath does not continue over the fan-shaped or bulb-like ending. The plasma membrane of the axon lies directly on the basement membrane of the smooth muscle cell. The width of the basement membrane, approximately 0.2 μ , thus separates the nerve ending from the muscle cell. The axoplasm of the nerve contains numerous vesicles about 30 m μ in diameter similar to those described as synaptic vesicles.

Observations on the myelinated nerves in the suprachoroid have been scanty because of the difficulty of obtaining well-preserved nerves. Damage occurs to them during dissection of the choroid from the sclera. A few specimens were obtained which show the myelin sheaths to be fairly well preserved (fig. 7). The nerve bundle is oval or flattened in the plane of the sclera. The myelinated nerves in the bundle are 7 to 15 μ in diameter and the Schwann cell nuclei are located at the periphery of the bundle. Some bundles contain both myelinated and unmyelinated nerves.

Melanocytes frequently are seen in close association with nerves, both in the suprachoroid and the choroid. A melanocyte may be stretched out along a side of a nerve bundle or some branches may completely encircle the bundle. At the nodal points of the nerve plexuses a melanocyte may be seen in the center of the plexus. In all these instances, the melanocyte can be demonstrated to be separated from the nerves by the thickness of the Schwann sheath and its basement membrane. In only one instance have we seen a contact between a melanocyte and a nerve (fig. 8).

Ganglion Cell. The most outstanding nerve structure in the choroid is the ganglion cell (fig. 9). This large neuron measures more than 25 μ wide at the perikaryon and may contain one or two nuclei. The nuclei are approximately 10 μ in diameter and are surrounded by the usual double membrane.

The cytoplasm contains numerous slender dense mitochondria measuring 0.8 to 1.5 μ

in length and 0.13 to 0.2 μ in width. The internal double membranes form finger-like projections which give the appearance of a vesicular internal structure to some of the mitochondrial profiles.

Throughout the cytoplasm there is a dense concentration of vesicles, lamellae, and granules in various forms. Even though organized endoplasmic reticulum is scarce, short lamellar segments and vesicular forms are abundant. There also are numerous smooth-surfaced vesicles, dense-rimmed vesicles, various vesicles containing one or more dense granules, and multivesicular bodies of varying densities. Most cells contain large homogeneous inclusions which are closely associated with irregular dense granular bodies.

The Nissl's bodies are large areas in the cytoplasm which contain many rosettes of dense ribonucleoprotein granules. The Golgi complex is relatively extensive near the nucleus and consists of short membranous canaliculi, small vesicles, and vacuoles. RNA-rich granules are absent from this zone.

Numerous small nerve fibers are in inti-

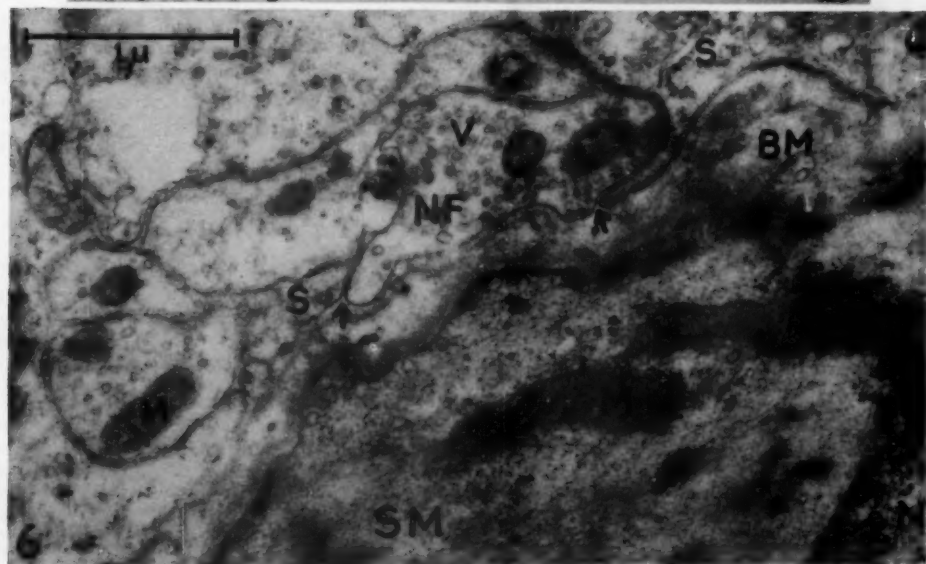
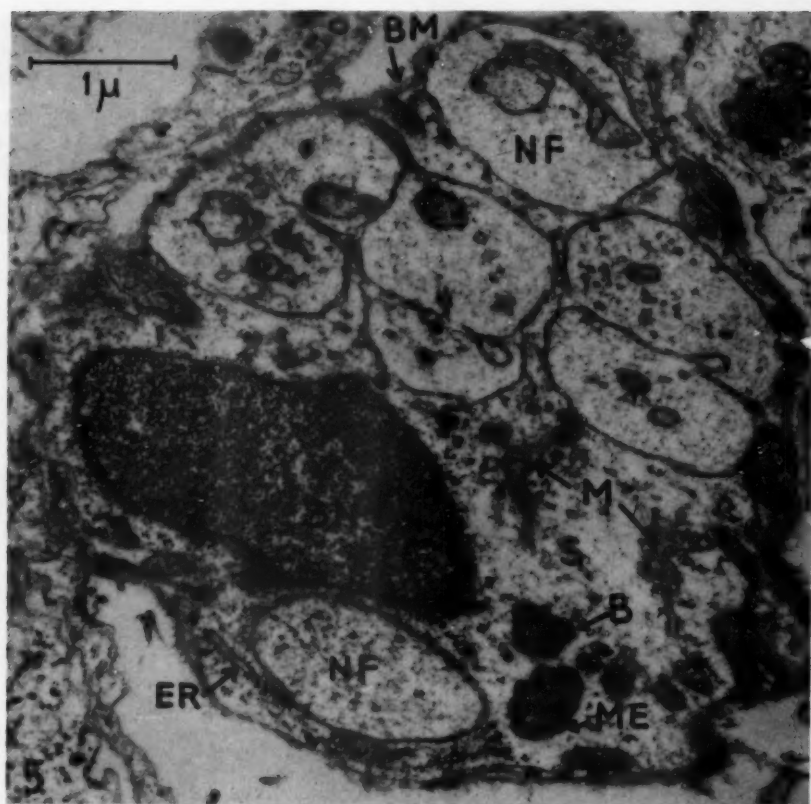


Fig. 5 (Feeney and Hogan, II). Small nerve bundle in the choroid consisting of eight nerve fibers and their Schwann cell. The cytoplasm of the Schwann cell (S) contains mitochondria (M), lamellae of endoplasmic reticulum with adherent RNA-rich particles (ER), a clump of melanin particles enclosed in a membrane-bounded vacuole (ME), and a vacuole containing granular material and a homogeneous body (B). The nerve fibers contain mitochondria, neurofilaments, and small vesicles.

$\times 22,000$

Fig. 6 (Feeney and Hogan, II). Electron micrograph of a nerve ending on the basement membrane of a smooth muscle cell of an artery. Cytoplasm of the Schwann cell (S) covers the nerve fiber (NF) except for the area between the arrows. The bare axon lies on the basement membrane (BM) of the smooth muscle cell (SM). The nerve ending contains numerous small "synaptic vesicles" (V). Mitochondria, M. Nucleus, N.

$\times 32,000$



mate contact with the plasma membrane of the ganglion cell (fig. 10). These fibers frequently produce deep indentations of the membrane. They generally are less than 0.8μ in diameter and they occur singly or in groups of a dozen along the surface of the ganglion cell. The nerve fiber may be separated from the ganglion cell by a thin layer of Schwann cell cytoplasm. Often the fiber is in direct contact with the ganglion cell, with only an intercellular space ($20 \text{ m}\mu$) and the respective plasma membranes separating them.

The nerve fibers which lie along the ganglion cell surface may be as small as $70 \text{ m}\mu$ in diameter; however, the average size is 0.5μ (placing most of them beyond the view of the light microscope). The axoplasm occasionally contains a mitochondrion, but generally mitochondria are absent. Dense bodies 0.1μ in diameter are frequently present. Vesicles of approximately $25 \text{ m}\mu$ size either may be seen in great numbers in a single nerve fiber, or may be completely absent.

Large numbers of nerve fibers usually are seen at one side of the cell and only a few around the remaining surface.

DISCUSSION

Most studies of the nerve supply to the choroid indicate that soon after entry to the suprachoroidal layer, the posterior ciliary nerves branch repeatedly and gradually lose their myelin sheaths. This study shows that even the first branches into the outer choroid are without myelin sheaths.

Bietti² and Wolter⁴ reported nerves in the innermost layers both in and beneath the lamina vitrea, but we are unable to find them at this level.

Nerve endings containing large (65 to $90 \text{ m}\mu$) dense vesicles, as well as endings containing small (23 to $30 \text{ m}\mu$) vesicles have been seen by us in thin sections of choroidal nerve bundles. Small vesicles have been found at neuromuscular junctions and nerve terminals in many parts of mammalian cen-

tral nervous system and have been classed as "synaptic vesicles."^{6,7,8} Larger vesicles (100 to $150 \text{ m}\mu$) have been shown to be a distinctive feature of the neurohypophysial nerve terminal and correspond to the neurosecretory granules seen by light microscopy.⁹ The significance of their presence in choroidal nerve endings is not known.

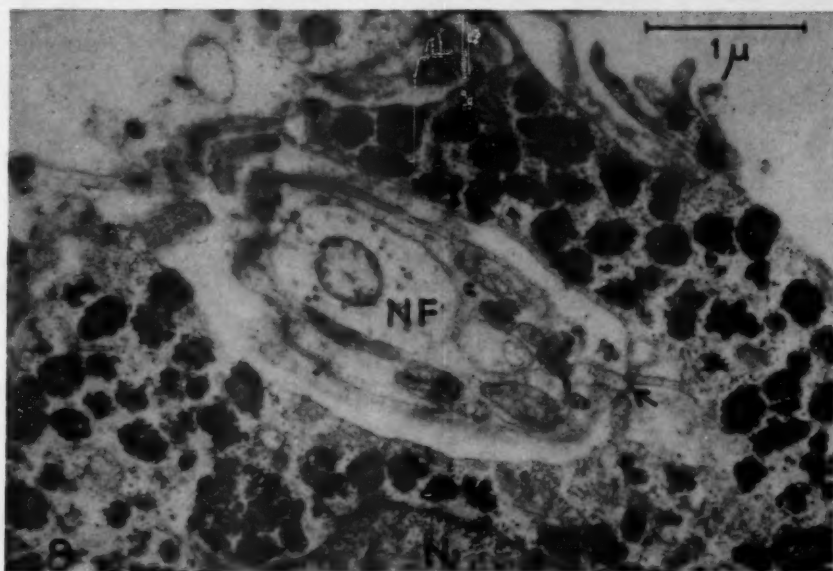
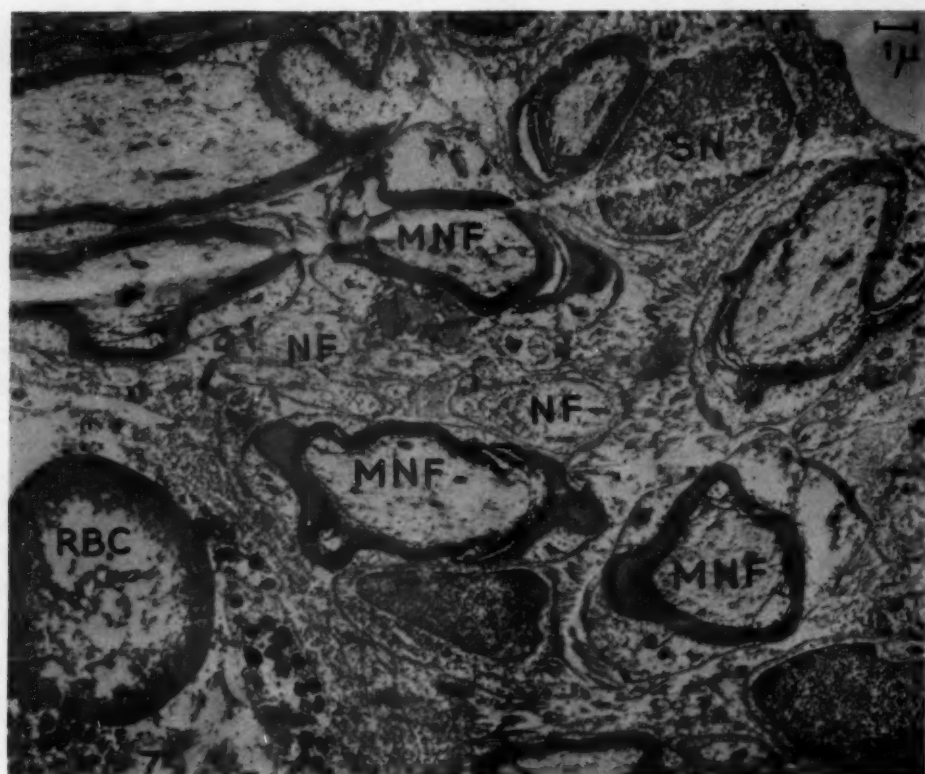
Those nerve bundles which come to lie in the adventitia of arteries and arterioles often have only three or four axons. Direct contacts between these fibers and the smooth muscle of the vessel wall are difficult to demonstrate. Sections from three of the eyes have shown a contact with the basement membrane of a smooth muscle cell, but not with the sarcolemma. Nerve endings of this type have been demonstrated by Ceasar et al.¹⁰ on the smooth muscle of the urinary bladder, but none were found by Pease and Molinari¹¹ on the muscle cells of small pial arteries.

The ganglion cells in the choroid are much more numerous than we had anticipated from studies of histologic sections and a perusal of the literature. The significance of this abundance is not clear, but presumably the ganglion cells are involved in mediation of the nerve impulses to the blood vessels. The ganglion cells are similar to those of the superior cervical ganglion¹² and the ciliary ganglion.¹³ A polar arrangement of



Fig. 7 (Feeney and Hogan, II). Nerve bundle in the suprachoroid containing myelinated (MNF) and unmyelinated (NF) nerve fibers. Schwann cell nuclei (SN). $\times 4,000$

Fig. 8 (Feeney and Hogan, II). Electron micrograph of a small nerve bundle surrounded by a choroidal melanocyte. Numerous dense melanin granules fill the cytoplasm of the melanocyte. Two fingers of cytoplasm extend in a pincer, contacting a finger of cytoplasm of the nerve bundle at the arrow. $\times 24,000$



axons on the ganglion cell is common, with 10 to 50 axons in contact with or embedded in the plasma membrane of the cell on one side, and only a few in contact around the remaining surface of the cell. The fine structure of the synapses is still under investigation.

The Schwann cell nuclei tend to be concentrated at the nodal points of the nerve plexuses, and their cytoplasm extends out widely over the axons in the bundles. Practically all the nerve fibers have only a single thin covering of Schwann cell cytoplasm. This arrangement is characteristic of all unmyelinated nerves.^{14, 15, 16}

The close alliance of melanocytes to nerve fibers in the choroid suggests the pigmented cells have a direct nerve supply. Very little evidence either for or against this hypothesis is supplied by this study.

San Francisco (22).

Fig. 9 (Feeney and Hogan, II). Electron micrograph of a portion of a ganglion cell of the choroid. Many nerve fibers (NF) are seen at one end of the cell and only a few around the remaining surface. Outlined areas represent Nissl's bodies. $\times 6,000$

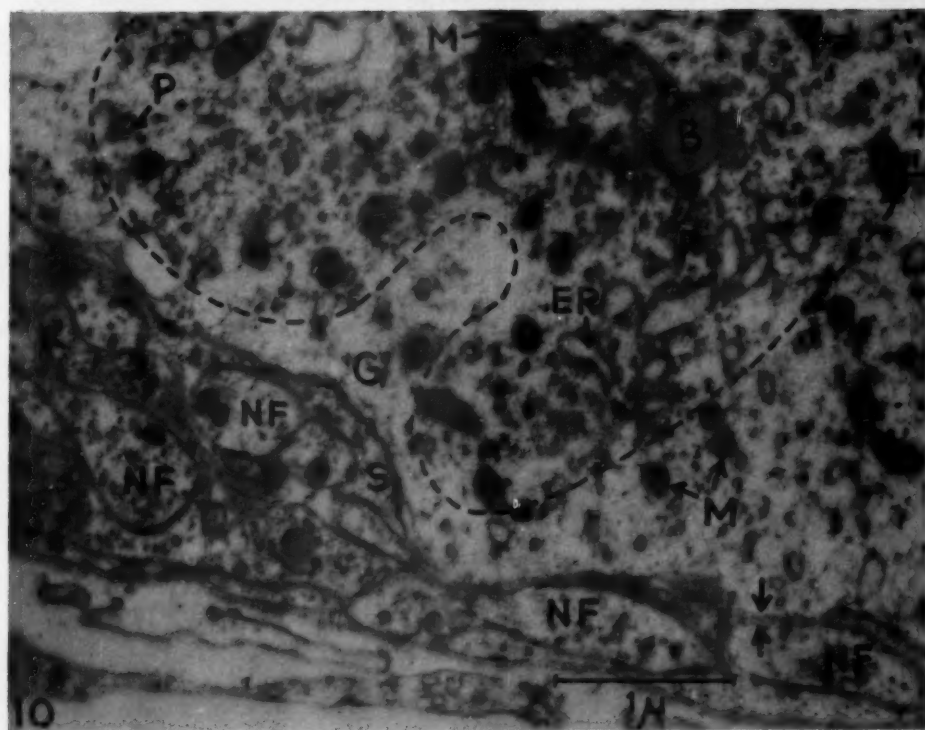
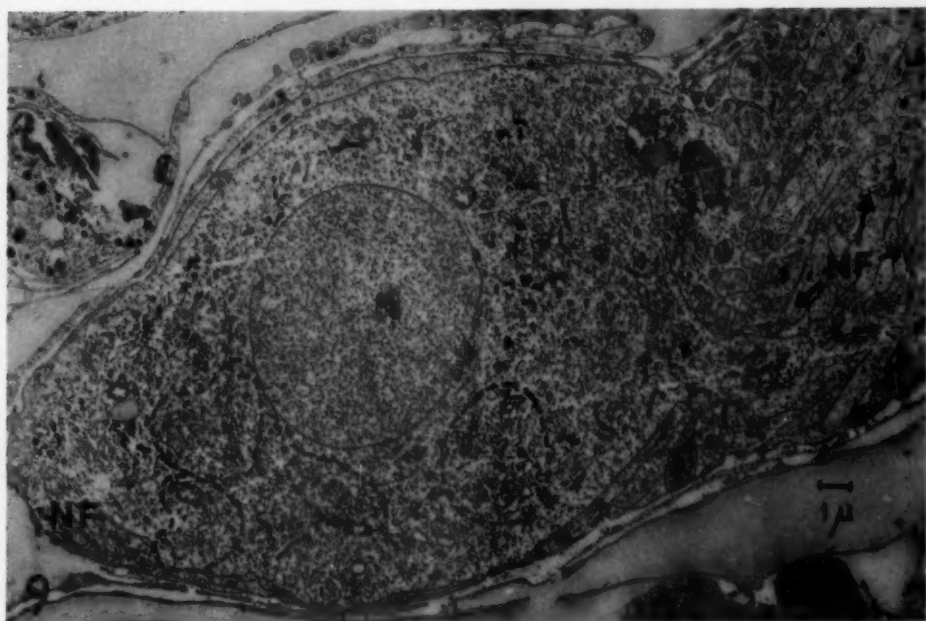
Fig. 10 (Feeney and Hogan, II). Electron micrograph of the periphery of a ganglion cell showing the relationship of the nerve fibers to the cell. A thin layer of Schwann cell cytoplasm (S) may separate the nerve fiber (NF) from the ganglion cell (G). The nerve fiber may be separated from the ganglion cell only by their respective plasma membranes (arrows).

The cytoplasm of the ganglion cell contains many slender dense mitochondria (M). The endoplasmic reticulum (ER) appears as membranous vacuoles studded with dense RNP particles. Many free particles (P) are also present. Two homogeneous dielectronic bodies (B) are seen associated with a mass of dense granular material. Enclosed area represents a Nissl's body. $\times 26,000$



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ELECTRON MICROSCOPY OF THE HUMAN CHOROID

III. THE BLOOD VESSELS

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INTRODUCTION

The vessel layers form the bulk of the structure of the choroid. The principal arterial supply is derived from approximately twenty short posterior ciliary arteries. They penetrate the sclera around the optic nerve, and enter the suprachoroid. Leber,¹ Ashton,² Vilstrup,³ and Wybar^{4,5} provided evidence that the anterior choroid is supplied also by recurrent branches from the long posterior ciliary arteries, through the major circle in the corona ciliaris. However, Francois,⁶ after study of microradiographic preparations, denies the presence of small arterioles or arteries in the pars plana which could anastomose with the anterior branches of the short ciliary arteries.

The initial branches of the short ciliary arteries lie in the suprachoroid. As the vessels pass anteriorly they divide continuously and enter the deeper stroma of the choroid, finally terminating in the capillary net. Generally the larger vessels lie in the outer layers, and the smaller ones in the mid- and deep stroma. Occasionally, however, reasonably large arteries are seen in the inner layers, especially in the posterior choroid. All the arterial and venous channels in the anterior choroid are of small caliber. The best evidence indicates that there are no arteriovenous anastomoses, except at the choriocapillary level.

In the posterior choroid the number of

veins coming out of the capillary layer is unusually large, and they seem to be slightly thicker than the corresponding arteries, but are just as tortuous. Anterior to the equator they become less numerous and more delicate. The venules and small veins are continuous with larger veins which have long, tortuous courses, as they pass toward the vortices.

Salzmann⁷ showed the walls of the choroidal arteries were of the same construction as those of small arteries with a plainly developed muscularis. Wolfrum⁸ showed the arteriolar smooth muscle cell was polymorphous, and branched, to surround the vessel in a patchy polypoid fashion.

A brief review of the normal anatomy of blood vessels is necessary before commencing a discussion of the results of our studies. The average capillary is 8 μ in diameter.[†] One to three endothelial cells encircle the lumen, being arranged parallel to the long axis. In some tissues, such as the choriocapillaries of the choroid and the renal glomerulus there are gaps between some of the endothelial cells. The endothelium lies on a thin basement membrane. A thin collagenous tissue forms a sheath around this membrane. Fixed macrophages and nerve cells often lie in this sheath, although in the human eye the capillary layer seems to be devoid of nerves.

The arteriole generally measures less than 0.3 mm. in diameter. It has an endothelium which is similar to that of the capillary. A thin internal elastic lamina lies outside the basement membrane of the endothelium. This layer disappears when the arteriole

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[†] Taken from Maximow, A. A. and Bloom, W.: *Textbook of Histology*, 7th ed., W. B. Saunders Company, Philadelphia, 1957.

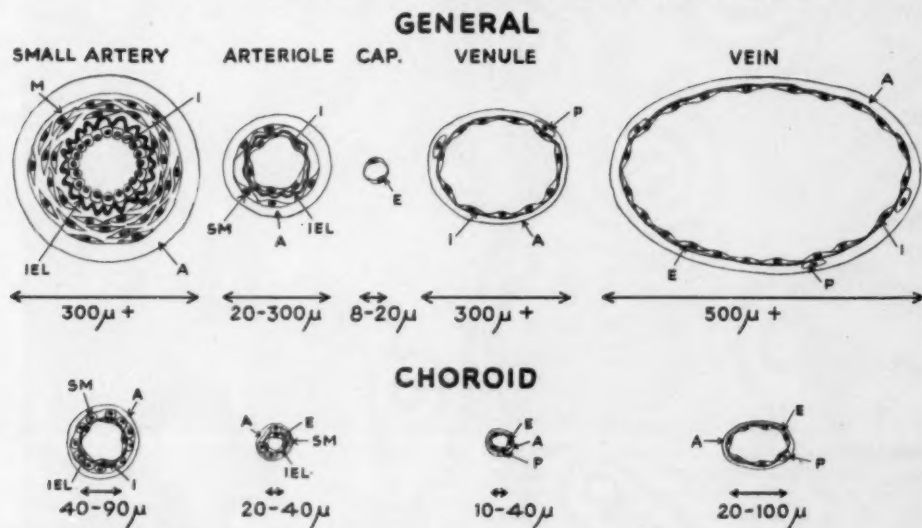
reaches about $62\ \mu$ in diameter. The muscle cells are continuous around the wall in the larger arterioles, and become fewer in number and more scattered as the vessels diminish in size. The individual cells are about 15 to $20\ \mu$ long. The adventitia contains bundles of collagen arranged parallel to the axis of the vessel, some elastic tissue, and occasional fibroblasts. The elastic tissue in the adventitia is lost at the capillary level.

The *small arteries* of the muscular type which are found in the choroid have an endothelium similar to that of the capillary, and a well developed internal elastic membrane which is homogeneous and bright by light microscopy. It usually is scalloped because of contraction of the muscle layer dur-

ing fixation and quite often is split, especially in larger vessels. The media contains a number of layers of smooth muscle cells, arranged concentric with the lumen. The adventitia is composed of loose connective and elastic tissue, connective tissue cells, and occasional macrophages.

Small *veins* are about $20\ \mu$ in diameter, and consist of a layer of endothelium surrounded by a layer of longitudinally directed collagen and some fibroblasts. Partially differentiated muscle cells may lie outside the endothelium of veins of about $50\ \mu$ diameter. Larger veins ($200\ \mu$ or more) have a continuous muscularis, composed of elongated spindle cells. The tunica adventitia shows scattered fibroblasts and thin elastic and

COMPARATIVE SIZE OF ARTERIAL AND VENOUS CHANNELS CHOROID AND GENERAL SYSTEM



P - PERIVASCULAR CELL
E - ENDOTHELIUM
A - ADVENTITIA
M - MEDIA
I - INTIMA
IEL - INTERNAL ELASTIC LAYER
SM - SMOOTH MUSCLE

Fig. 1 (Hogan and Feeney, III). Schematic drawing showing the comparative structure and size of choroidal vessels and those of other parts of the body.

collagenous fibers running longitudinally.

The following table illustrates some of the differences between choroidal vessels and those in other parts of the body (fig. 1).

METHODS AND MATERIALS

The sections for this study were obtained from four human eyes fixed immediately after enucleation, embedded in plastic, and cut as described previously.⁹

Arteries. Measurements were made of the diameters of the lumina of the posterior choroidal arteries after the first branches in the suprachoroidea. The larger vessels average $35 \times 60 \mu$ with a maximum of $40 \times 90 \mu$. Smaller stromal arteries average $25 \times 40 \mu$ with a maximum of $27 \times 50 \mu$. These measurements are somewhat less than in the living state, however, because of the contraction produced by the muscularis.

The endothelial cells lining the choroidal arteries form a single layer. They vary in

thickness depending on the state of contraction of the artery, but generally are about 5μ thick in the nuclear region of the cell and about 0.4μ thick in the thinner portions. Adjacent cells overlap slightly or show a simple interdigitation. The nuclei either may be flattened or may protrude into the lumen. The nucleolus is prominent and the nucleus is surrounded by the usual double membrane. The cytoplasm contains fine fibrils, and numerous free granules, possibly of RNP. There are some lamellar and vesicular elements of the endoplasmic reticulum. The mitochondria are found mostly in the nuclear region. Pinocytotic vesicles are seen near the plasma membrane on the side of the lumen as well as on the outer side, facing the basement membrane (fig. 2A, B).

The endothelium lies on a basement membrane which blends into, and at times is indistinguishable from, the internal elastic lamina. The basement membrane however,

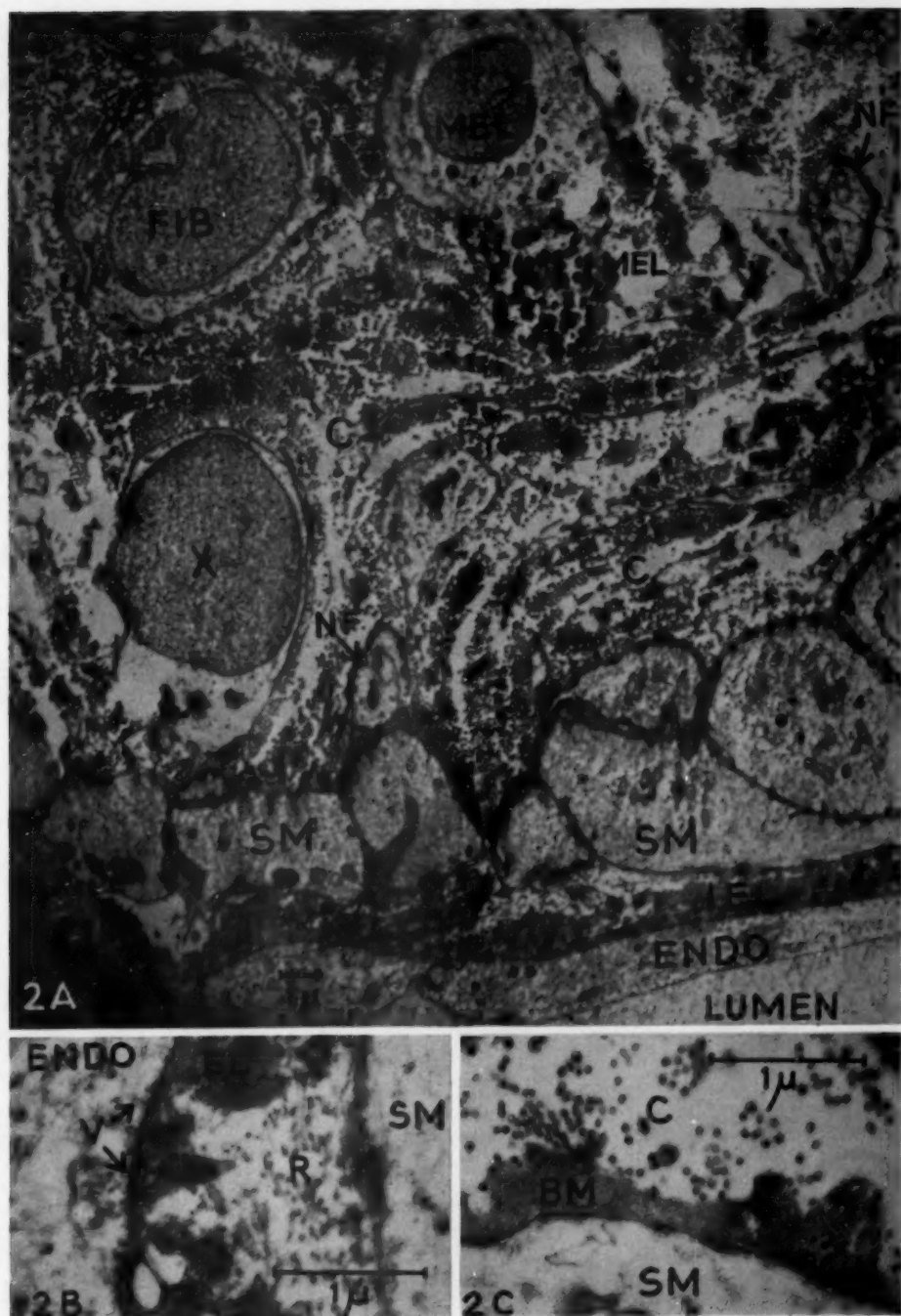
Fig. 2A (Hogan and Feeney, III). Electron micrograph of the wall of a posterior choroidal artery and adjacent tissue. Endothelial cells (ENDO) line the lumen. The internal elastic lamina (IEL), the muscularis (SM), and the adventitia lie external to this. The adjacent tissue contains numerous collagen fibers (C) seen in cross-section, nerve fibers (NF), a sparsely pigmented melanocyte cut through its nucleus (MEL), a portion of a heavily pigmented melanocyte (MEL), part of a fibrocyte sectioned through its nucleus (F, 1B), and a large unidentified cell (X) which differs from the muscle cells by having a "clear" cytoplasm (no myofibrils). $\times 7,500$

2B and 2C compare the size of the fibers in the

internal elastic lamina and those occurring in the adventitia.

2B. The elastic lamina contains elastic tissue (EL), and reticulin fibers (R) with diameters about one-half the size of the collagen fibers of the adventitia. The endothelial cytoplasm (ENDO) contains pinocytosis vesicles (V) which open onto the elastic layer. Smooth muscle cell (SM). $\times 24,000$

2C. The diameter of the collagen fibers (C) should be compared with reticulin fibers (R) in figure 2B. A thick basement membrane (BM) separates the smooth muscle cell (SM) from the adventitia. $\times 24,000$



seen at high magnification, appears to have a fine fibrillar structure which is not seen in the elastic layer.

The internal elastic membrane is composed of elements which appear as homogeneous areas of lighter density than the basement membrane material. It frequently contains dark bodies which were first noted by Fawcett,¹⁰ who considered them to be due to a pathologic change. Fenestrations are seen in the elastic lamina. These appear as clear dielectronic areas and frequently contain fine collagen fibrils. The collagen fibrils in this position measure 20 to 30 m μ in diameter (fig. 2B), while those in the adventitia measure 40 to 50 m μ in diameter (fig. 2C). According to Fawcett, the smaller fibers are identical with the argyrophilic reticulum of silver-stained histologic sections which is thought to be composed of reticulin fibers.

The basement membrane of the muscle cell is more easily distinguished from the internal elastic lamina than is that of the

endothelium (fig. 3). The membrane surrounds all surfaces of the individual muscle cells. The basement membrane either may be shared as a single layer between two muscle cells or two basement membranes may be seen back-to-back (fig. 4A). At times a space containing fine collagen fibrils separates the two basement membranes (fig. 4B).

Many small collagen fibers (reticulin) are found at the triangular junctions formed by the apposition of two or more muscle cells, and these generally are embedded in a dielectronic ground substance rather than in the basement membrane. The basement membranes facing the adventitia or choroidal stroma may be more than 0.1 μ wide while those occurring between muscle cells may be only 0.04 μ in thickness.

The muscle cells are arranged longitudinally along the vessel as a single layer. The outer surface of this layer, facing the choroidal stroma, presents a scalloped surface. The nucleus is oval and flattened, but

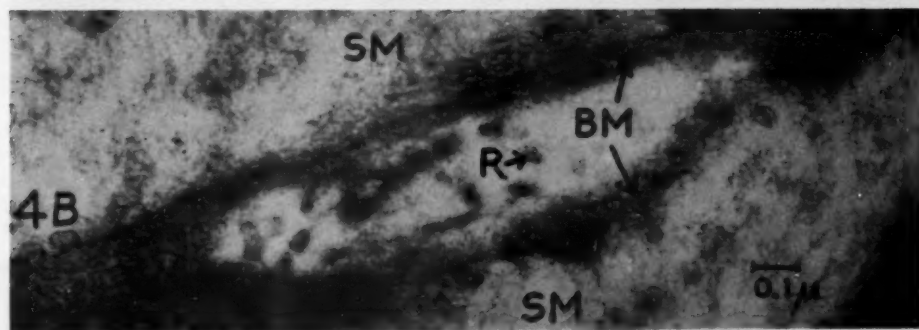
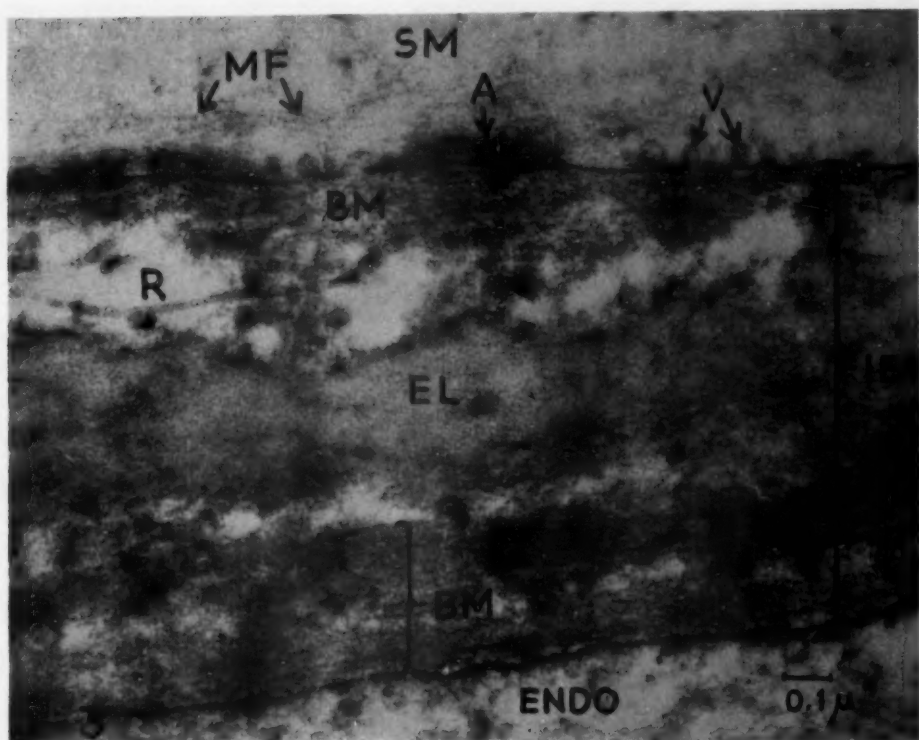
Fig. 3 (Hogan and Feeney, III). Electron micrograph of the internal elastic lamina (IEL) of a choroidal artery. The basement membrane (BM) of the endothelium (ENDO) is distinguishable from the elastic tissue (EL) by its more fibrillar texture. A dielectronic space containing a reticular fiber (R), separates the elastic tissue from the basement membrane of the muscle cell (SM). Myofibrils (MF) and their areas of attachment to the plasma membrane (A) may be seen in the cytoplasm of the smooth muscle cell. Pinocytosis vesicles (V) line the plasma membrane facing the basement membrane. $\times 70,000$

Fig. 4 (Hogan and Feeney, III). Portions of two muscle cells and their basement membranes. $\times 70,000$

4A. (SM) smooth muscle; (V) pinocytosis vesicle; (A) attachment areas for myofibrils; (BM) basement membrane.

4B. The same, showing splitting of the basement membranes of two cells. The space contains reticulin fibers (R).

4C. Somewhat flat section through the edge of a smooth muscle cell, showing the numerous vesicles (V).



appears round when the artery is sectioned transversely. The cytoplasm contains a Golgi complex, mitochondria and membranous vacuoles studded with granules, possibly of RNP. Free granules in the cytoplasm may represent the RNA-rich granules or glycogen. There are numerous myofilaments throughout the cytoplasm which gives the cell a strikingly dense appearance compared to other choroidal cells. Dense rectangular nodes are seen at intervals around the inner surface of the plasma membrane, seeming to be produced by the numerous myofibrils which converge to attach to the plasma membrane.

Pinocytosis vesicles about 40 m μ in diameter are abundant around the entire inner surface of the plasma membrane of the smooth muscle cells (fig. 4C). These vesicles presumably are engulfing or drinking fluids and nutritive material from the intercellular matrix. Fawcett observed that "one may infer from this that the intercellular matrix does not constitute a significant barrier to the passage of solutes from the lumen into the vessel wall."

The nerve synapses which we have observed on these muscle cells have been described in a previous paper.¹¹

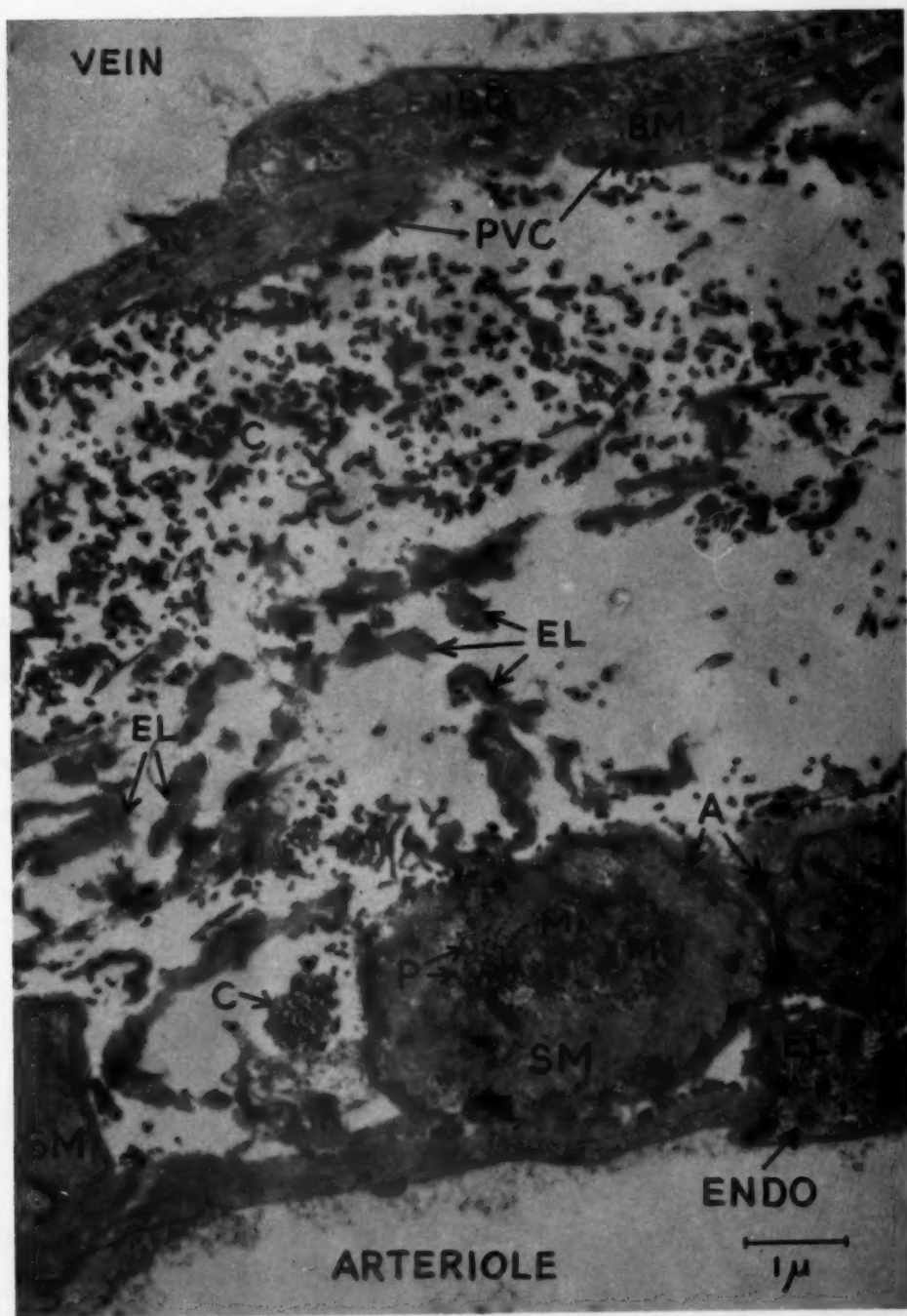
The periphery of the muscle cell is surrounded by a relatively thick basement membrane, which separates the cells from the adventitia. An external elastic lamina is not seen. The adventitia is composed of bundles of collagen fibers and scattered elastic fibers. Occasional fibrocytes are seen.

At times a cell resembling muscle cells is seen just outside the muscularis (fig. 2). The cytoplasm of this type of cell is less dense and lacks the typical myofibrillar pattern of the true muscle cell.

Arterioles. The average diameter of the lumen of the arteriole is 29 μ with a minimum of 21 μ and a maximum of 42 μ . An arteriole is recognized by the diminution and disappearance of the internal elastic lamina and discontinuity of the muscularis (fig. 5). The elastic tissue disappears first, leaving only the two broad basement membranes of the endothelium and the muscle cells. These basement membranes gradually narrow to a layer of 60 m μ or so. Finally a muscle cell is lacking here and there along the vessel wall (fig. 6). Arterioles may have muscle cells on one side of the lumen and not on the other. The muscle cells and endothelium appear identical to that of the artery. The collagen of the arterial ad-

Fig. 5 (Hogan and Feeny, III). Electron micrograph showing a portion of the walls of a vein and of an arteriole. The vein wall consists of the endothelium (ENDO) and a homogeneous basement membrane (BM). Cytoplasmic extensions of perivascular cells (PVC) are seen next to the basement membrane. The choroidal stroma consists of a ho-

mogeneous ground substance in which collagen (C) and elastic (EL) fibers are embedded. The wall of the arteriole consists of an endothelium, a thin elastic lamina (IEL), and an intermittent smooth muscle layer (SM). The muscle cell shows myofibril attachment zones (A); mitochondria (M) and numerous dark, fine RNA-rich particles (P). $\times 16,000$



ventitia diminishes considerably in the arteriole.

Veins. Measurements of the diameters of the lumina in veins of the outer posterior choroid show great variations in size. The largest measure $30 \times 130 \mu$ and many measure about $20 \times 100 \mu$. Those in the stroma measure $20 \times 30 \mu$ in size. The venules in the innermost regions measure from $5 \times 7 \mu$ to $10 \times 40 \mu$. It is difficult to obtain accurate cross-sectional measurements of veins because of their extreme tortuosity. The large veins approaching the vortices are not included in these measurements.

The wall of a vein as seen in thin sections generally consists only of a thin layer of endothelium and a basement membrane (fig. 7A, B). There usually is very little collagen immediately outside the basement membrane. No muscle cells are seen in the walls of the choroidal veins. Supporting cells (perivenous cells or pericytes) are seen but large areas along the vessel wall show no cells. These cells may be closely applied to the wall, their basement membranes merging with that of the endothelial cell, or there may be a connective tissue space between them. Occasionally the perivenous cell may lie directly against the endothelial cell. In such a case the basement membrane of the

endothelium is continuous with a membrane enclosing the perivenous cell. Cells resembling lymphocytes frequently are seen just outside the walls of small veins. The small veins and venules which lie just beneath the choriocapillaris appear to have more perivenous cells on their outer walls than do the other veins. The cytoplasm of the perivenous cells contains the usual organelles. No fibrils (tonofibrils) have been demonstrated. Nerve contacts have not been demonstrated with certainty, although one micrograph strongly suggests its occurrence (fig. 8).

True capillaries are not seen in any layer of the choroid except the choriocapillary layer. The small veins in the inner choroid beneath the choriocapillary layer have an identical structure to the capillaris but the caliber is much larger.

DISCUSSION

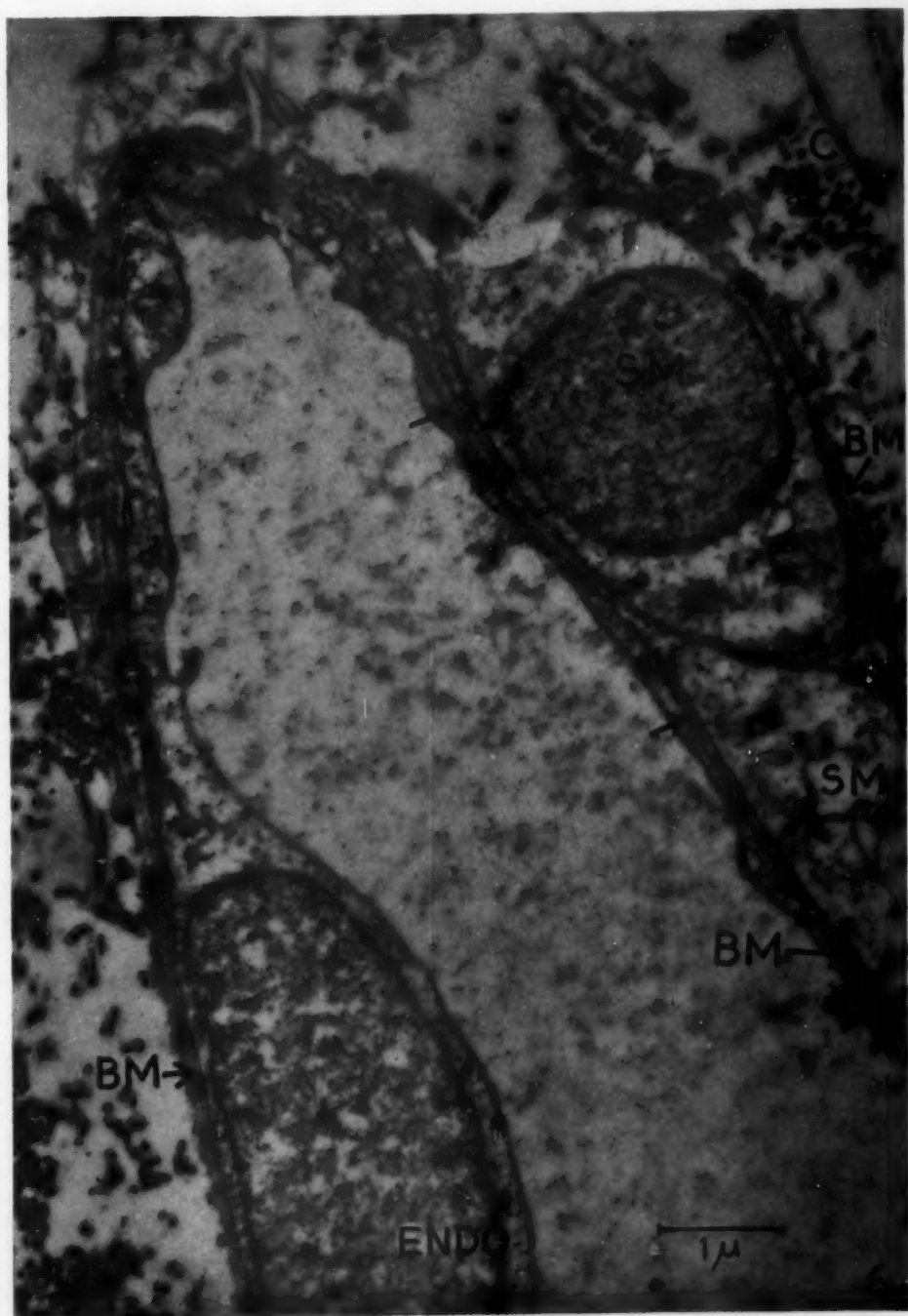
The small arteries and arterioles of the choroid seem to be identical to those described by other authors in other tissues of humans and animals.^{10, 12, 13}

The abundance of myofibrils in the cytoplasm of the muscle cells in the choroidal arteries is noteworthy. A number of observers have described nodular thickenings in the plasma membrane of these cells.

Fig. 6 (Hogan and Feeney, III). Electron micrograph of a portion of an arteriole. Smooth muscle cells (SM) are present on one side of the vessel and absent from the other. The nucleus of an endothelial cell (ENDO) is surrounded by cytoplasm. The base-

ment membrane (BM) of the endothelial cell faces the choroidal stroma. Two basement membranes lie side by side at (BM) (arrows). One belongs to the muscle cell and the other to the endothelial cell.

×19,000



Pease and Molinari¹² were the first to suggest these thickenings provided the attachments for the myofibrils and a fixed point from which contraction could occur.

Connections of nerve axons to the basement membrane of arterial smooth muscle cells are of great interest. The connections we observed were not specialized, the axon coming in contact with the basement membrane of the muscle cell. The extraordinary number of nerves in the choroid, and their intimate relationship to the arteries would suggest that many such connections might be observed. Pease and Molinari¹² studied the arteries of the pia mater in the cat and monkey and found no direct nerve connections to the muscle cells. Such connections, however, have been observed in the smooth muscle of the urinary bladder by Caesar, et al.¹⁴

Contact between adjacent muscle cells through the rather heavy basement membrane has been described by a number of observers.^{15,16} We found occasional extensions of the plasma membrane through this basement membrane. Direct cytoplasmic connection between the two cells, however, was

not observed. It is suggested these connections either bring the cells into closer contact to carry impulses concerned with contraction or serve to share nutritive material. Occasionally we find projections of the endothelium through the basement membranes and elastic layers to make contact with the muscle cells. These are similar to those observed by Moore and Ruska.¹⁷

A double muscle layer is not seen on any of the choroidal arteries, such as was described by Wolter.¹⁸ Also, we found the muscularis to be continuous in these vessels, contrary to the observations of this author.

We have sectioned many parts of the choroid in these four eyes, and at no place have we observed specialized shunts between arteries and veins. In this respect we agree with Ashton.² Connections between arterioles and the choriocapillaris often are abrupt, and their connections have large openings.

Studies of the retinal pigment epithelium, Bruch's membrane and choriocapillaris of these eyes will be reported later by another observer in our laboratories.

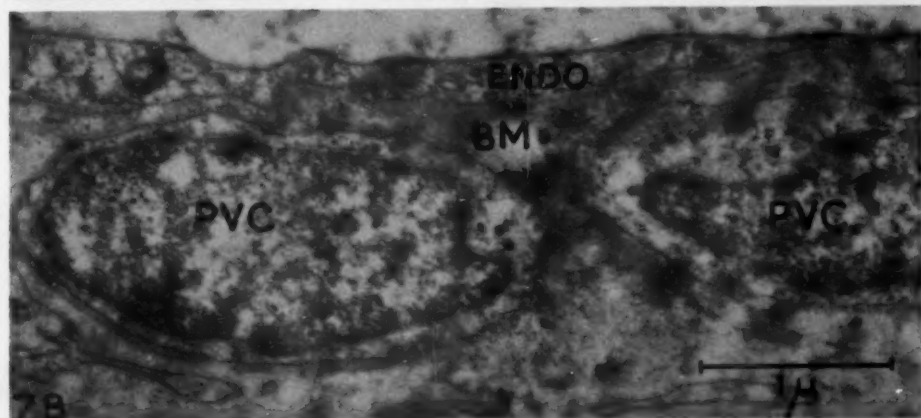
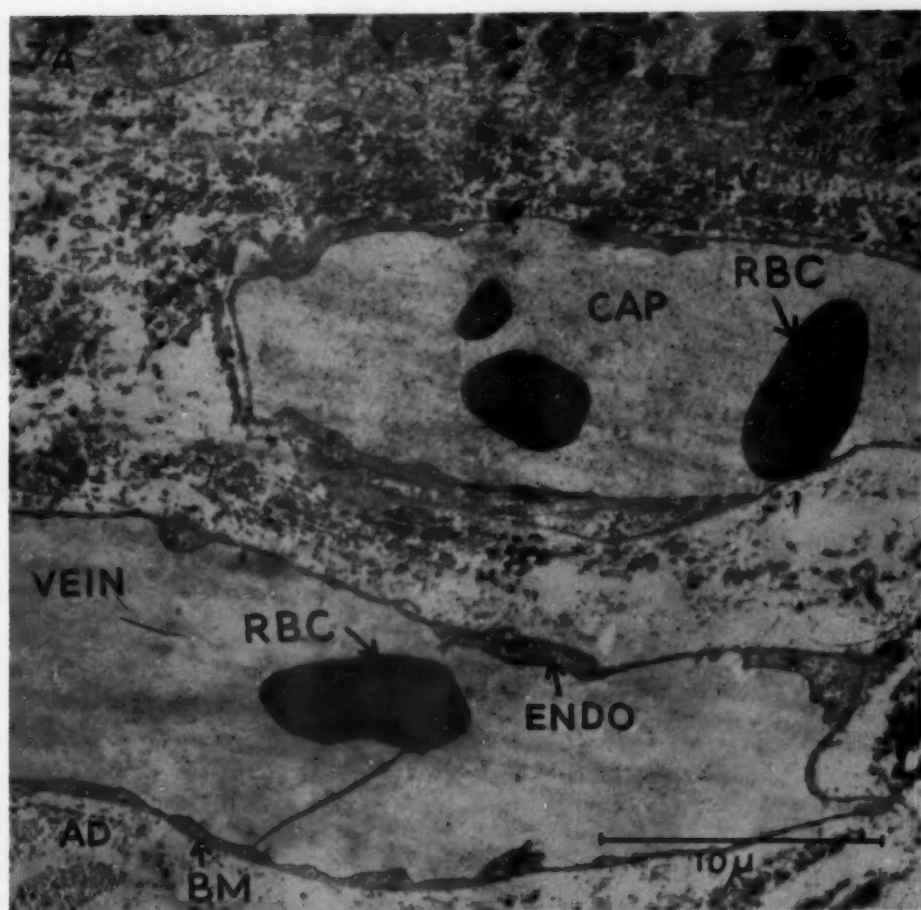
San Francisco (22).

Fig. 7 (Hogan and Feeney, III). Electron micrograph of a small vein in the inner choroid, and of a portion of the wall of a larger vein in the outer layers.

7A. Small vein. (ENDO) endothelium; (RBC)

red blood cell; (BM) basement membrane; (Ad) adventitia; (PE) pigment epithelium; (LV) lamina vitrea, (CAP) choriocapillaris. $\times 4,300$

7B. Large vein. (PVC) perivenous cell. $\times 29,000$

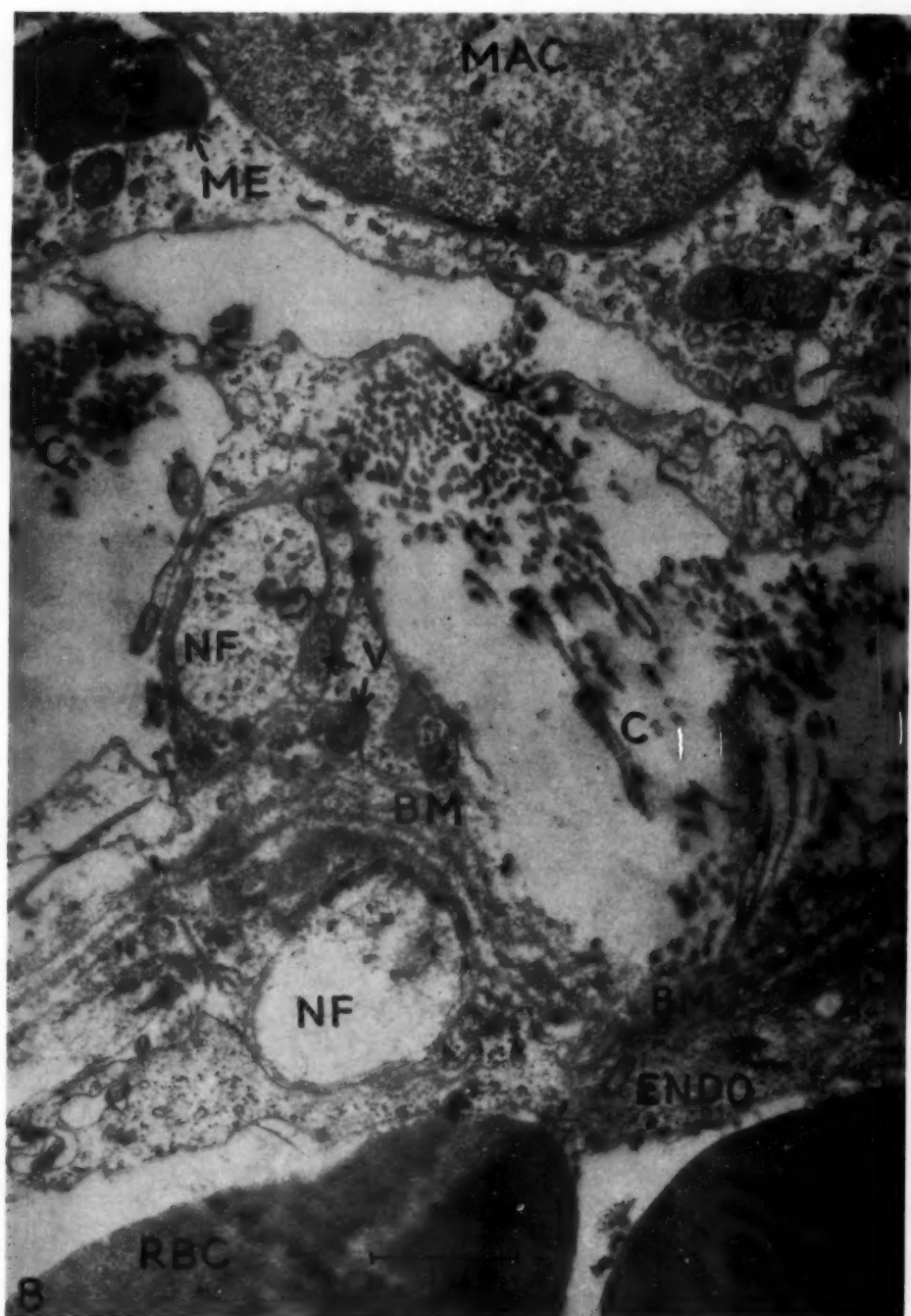


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Fig. 8 (Hogan and Feeney, III). Large vein with nerve fibers in the wall. (ENDO) endothelium; (BM) basement membrane (cut obliquely, so that it appears layered); (NF) nerve fibers, some of which contain vesicles (V); (C) collagen on cross- and

longitudinal section; (MAC) macrophage; (M) mitochondria of macrophage; (I) inclusions in macrophage; (RBC) red blood cell; (ME) melanin granule in macrophage. x23,500



THE NATURE OF CONTRAST PHENOMENA

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The long history of color theory is marked by violent controversy. Much of the controversy centers about the phenomena of color contrast.

There are two principal types of color contrast, successive contrast and simultaneous contrast.

Successive contrast has also been called after images, local adaptation, successive induction, temporal induction, and images due to retinal fatigue. It occurs in the same area of the retina which has been stimulated and it requires some measurable amount of time for the stimulus to produce its effect. If one fixes his gaze at a colored area such as a red square for a period of 30 seconds and then looks away at a white field the complementary color will be seen, blue-green in the area that was stimulated by the red square. This is called a negative after image. If the original red square had been a bright light and the gaze had been shifted to a black background, a red stimulus or positive after image may be feebly seen. The negative after images are much more easily perceived than the positive. Proponents of all the principal color theories have had little difficulty in explaining after images in terms of persistence of the stimulus or in terms of retinal fatigue. As the after image fades it sometimes passes through a remarkable succession of other colors before it disappears entirely.

Craik,¹ in a very ingenious experiment, presented convincing evidence that the site of after images is in the retina. He exposed an eye to a light stimulus while simultaneously pressing on the eye hard enough to make it sufficiently anoxic so that no light perception was present. When the stimulus

was discontinued and the pressure was released ordinary after images were seen.

Simultaneous contrast, which is also called spatial induction, or lateral adaptation, can be defined as the reciprocal inter-action of contiguous or nearly contiguous areas of the visual field. No measurable amount of time is required for the appearance of the phenomena, it occurs instantaneously. In simultaneous contrast, the area immediately adjacent to the area stimulated is affected so as to be influenced towards the complementary color. For example, if a small gray area is surrounded by a red field, the gray area takes on a bluish-green tinge.

Explaining simultaneous contrast has led to violent controversy which has embroiled such important minds as Goethe, Schopenhauer, Helmholtz and Hering.² Helmholtz² believed that simultaneous contrast was an "illusion of judgment," that it was psychological; but he was strongly opposed by Hering² who furnished evidence that the excitation of one region of the retina modifies contiguous areas of the retina. Hess,² Linksz³ and others vigorously supported Hering and supplied convincing evidence that Helmholtz was wrong. But as recently as May, 1960 McCamy⁴ of the National Bureau of Standards, Washington, D.C., speaks of the two types of contrast phenomena as being "psychological effects" and states, "Actually these two effects are not easy to separate because when two colors are in the field of view, the eye scans back and forth so that successive contrast effects are operating."

Recently, controversy concerning simultaneous contrast has flared with renewed fury following a series of articles by Land.^{5,6,7} Land beautifully demonstrated some remarkable phenomena which although not new had received little attention.

His technique consisted first of taking two

*From the Division of Ophthalmology, Department of Surgery, Stanford University School of Medicine. Presented at the Midwinter National Meeting of the Association for Research in Ophthalmology, New Orleans, Dec. 6, 1960.

photographs on black and white film of the same multicolored scene, one picture being taken through a red filter and the other through a green filter. Using two projectors he superimposed the black and white positive transparencies of these pictures on a single screen; if a red filter was then placed in front of the projector containing the transparency originally photographed through a red filter the multicolored scene appeared in a variety of colors. If instead of white light a narrow band of pure yellow light were used in the other projector, colors such as green were still apparent on the screen. Because of his experiments Land postulated a new theory of color vision, but Walls⁸ and Judd⁹ have pointed out that Land's phenomena are largely based on simultaneous contrast.

Stimulated by Land's experiments I attempted to reproduce them. This necessitated using two projectors with a variable resistance attached to one of them so that the luminance could be varied, two positive transparencies, and an assistant to help with the difficulties of registration on the screen. Land's experiments could be reproduced this way, but the equipment was clumsy and difficult to handle. I therefore contrived a simple instrument which I called the "color-former" which would achieve a similar result using black and white printed pictures in a small device I could hold in my hand. With it the subject could be investigated many times more rapidly and without assistance. A more complete description of this instrument has been given in a previous communication¹⁰ so that it need be described only very briefly here. It consists essentially of a partially transparent mirror, a pair of black and white prints, one photographed through a red filter and the other through a green filter, the latter image printed so as to be inverted. A special stand holds the prints on opposite sides of the mirror and at equal angles with the mirror so that the images are superimposed. Color filters may overlay the prints or the light source may be changed

at will. By substituting colored cards or designs for the printed picture on one side and a white card for the print on the other side, images appear as if a bright white light had been thrown on the colored card. By turning the instrument slightly in relation to the light source the amount of light on either side of the mirror is readily varied over a wide range without the use of variable resistors or other special equipment.

As has been described in a previous paper,¹⁰ this instrument has not only proven to be useful for the study of contrast phenomena, but may prove to be invaluable as a convenient method of transmitting color information on black and white printed images and in a variety of other ways.

For example, black and white prints could be printed cheaply in a scientific periodical and could then be viewed with this device in color. Black and white pictures of scenes taken within the hour could be printed in our newspapers and viewed in color through the instrument, although the color would not be as true as a good color photograph. Prints of multicolored draperies or other designs could be rapidly viewed in a succession of a variety of contrasting colors to see which combination pleases the observer most. It is possible that red-green color blind individuals might enjoy looking at a series of prints of great paintings in which the dominant colors are red and green but in which the contrast between these colors was not previously apparent to them. With appropriate filters such as blue and yellow these individuals could immediately appreciate the contrast in the paintings, even though they did not appear red and green to them.

The value of being able to easily vary the intensity of the superimposed strong white light was suggested by an important experiment of H. Meyer¹¹ and by the experiments of von Goethe¹⁰ with his colored shadows.

Meyer's experiment is astonishingly simple. If a small gray area on a vivid red background is viewed it may or may not be noted that the gray appears faintly blue-green be-

cause of simultaneous contrast. If a sheet of tissue paper is then superimposed on the red field and gray spot and the latter is viewed through the white tissue paper, the gray area seems to be much more blue-green (almost vivid blue-green) although the red surrounding field is now almost white. The addition of white light from the tissue paper greatly enhances the simultaneous contrast effect even though the surrounding area becomes much less saturated.

Goethe¹² showed that shadows may have color. The shadow of an object illuminated by a single colored light will, of course, appear black; but if this shadow is illuminated by another white light it will have the complementary color of the original light causing the shadow.

Thus, a second white light superimposed on a multicolored field frequently enhances the simultaneous contrast effect. The optimum strength of the second light source can easily be determined with the instrument by trial.

Meyer's experiment and the second white light are the keys to the phenomena demonstrated by Land. Four experiments are demonstrated here by projection.

The first three of these are demonstrated by means of two projectors, although these experiments were originally done with the instrument mentioned earlier. Four significant items of information are revealed:

(1) *If the additional white light is applied only to the area in which color is to be induced, the simultaneous contrast effect is still enhanced.*

Two identical small red circular areas are placed side by side, one being surrounded by a blue field and one by a yellow field. When strong white light is superimposed upon the entire scene, the red spot on the blue background looks orange and the other red spot looks purple. If the strong white light is superimposed *only* on the red areas, the same changes occur but are less marked in degree.

(2) *The influence exerted by the imme-*

diately contiguous area is much greater than that exerted by a far larger area in the periphery which is not contiguous.

On a large red field a central, small gray area is placed surrounded by a green rim of color which is relatively small in comparison to the large red field. When strong white light is superimposed on the entire field, the central area appears reddish having been influenced by the surrounding green rim rather than the much larger red area in the periphery.

(3) *A narrow rim of white or black surrounding an area protects this area from the effects of induction from the area surrounding the rim.*

On a large red field three identical small gray circular areas are placed. Around one is a narrow black rim and around the second a narrow white surrounding rim. When the strong white light is superimposed only the gray area without a surrounding rim takes on the complementary blue-green color.

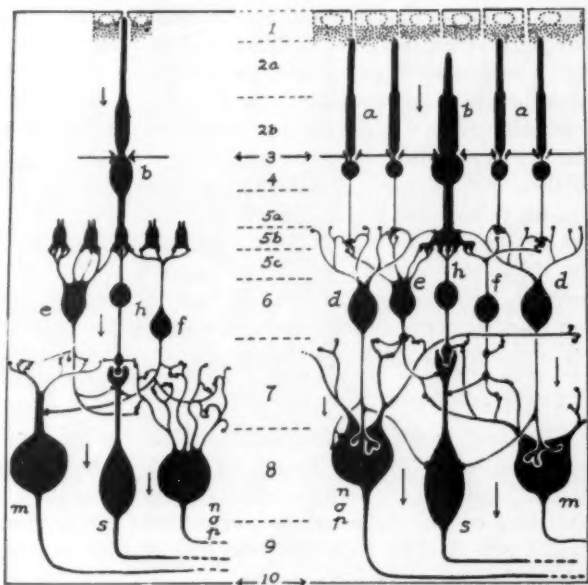
(4) *When visual angles are made progressively smaller chromaticity will be retained longer on a black background than on a white one.*

Four colored round areas or dots of equal size and different colors are placed on a black background on a card and four identical areas are placed on a white background on the same card. As one retreats from the card the visual angles of the colored areas become smaller, until a distance will be reached where the colors on the black background are still identifiable whereas those on the white background are not.

POLYAK

Polyak^{13,14} made a remarkable contribution in his anatomical studies of the anatomy of the retina. He minutely described at least three different types of bipolar cells, and a number of types of ganglion cells in the retina. On the basis of their position and synaptic connections he postulated that color discrimination is achieved chiefly by means of the bipolar and ganglion of the retina.

Fig. 1 (Flocks). Note that the bipolar cells are of several types (d, e, h, f) and synapse with more than one photoreceptor (a, b). From Polyak, Stephen L., *The Vertebrate Visual System*, Figure 178, edited by Heinrich Kluver, University of Chicago Press, Chicago, 1957. Copyright 1957 by the University of Chicago.



THE THEORY OF THE SHARING OF THE USE OF CHROMATIC ANALYZERS BY ADJACENT PHOTORECEPTORS

Based on experiments performed, and based on what is already known about simultaneous contrast, and aided by the findings of Polyak, an hypothesis has been proposed to explain the physiologic basis of simultaneous color contrast.

This new theory, in brief, is this: When an area of the retina is stimulated by light, chromaticity of the stimulus is analyzed by cells of the retina which serve not only the photoreceptors of the area that is stimulated, but which also serve the photoreceptors of the small area immediately adjacent to the stimulated area. If the immediately adjacent area is being simultaneously stimulated by another light, some of the chromatic analyzing cells which ordinarily serve it are being utilized by the original stimulus in the adjacent area of the retina and are therefore not available for analyzing the second stimulus. For example: If a small doughnut shaped area of the retina is stimulated by a red light, the chromatic analyzing cells for red that serve

the photoreceptors of the area of the retina stimulated are utilized as well as those of the immediately adjacent area; e.g., the chromatic analyzers that serve the area of the "doughnut" as well as its "hole" are used. If a white light is superimposed on the entire area or simply on the area of the "hole" of the "doughnut," the white light in the "hole" appears blue-green because the blue and green analyzers are free to respond, whereas the red analyzers are in use by the red stimulus to the surrounding area.

This new concept is fully in accord with the trichromatic theory of Young and Helmholtz.

It readily accounts for the phenomena of simultaneous contrast including those phenomena demonstrated by Land. The two pictures used by Land are taken through filters from opposite parts of the spectrum. If the transparencies are projected one with a red filter and one with a white light, an area that was originally red will allow red light to go through the red projector and no light through the other projector so that this area will appear red. An area that was originally green will allow no light to pass through the

red projector so that it would appear black except that when this black is illuminated by the white light which passes through strongly from the white light projector—the area appears blue-green by simultaneous contrast. Various combinations of colors are produced in this way by varying amounts of light passing through the two projectors in various areas of the picture. The appearance of the difference is not a psychological phenomena based on expectations. The colors can be reversed or changed through a variety of contrasting combinations.

THE SPREADING EFFECT OF VON BEZOLD

The new concept readily explains the spreading effect of von Bezold of which Evans¹⁵ states “. . . until the effect can be explained without elaborate assumptions, we cannot say that we understand the way in which the visual process operates.”

The spreading effect is simply this: If two identical spots of the same color are viewed, one being surrounded by a collar of white, and one by a collar or rim of black, the one being surrounded with black will appear a more intense or darker red than the one surrounded by white. Any color can be substituted for the red. The white surround seems to cause loss of saturation in the red spot and the black increases the saturation of the color and makes it appear darker. Evans¹⁵

states, “It need hardly be pointed out that the brightness changes are directly contrary to what would be predicted from our knowledge of simultaneous contrast. The presence of black borders in a picture should make the colors lighter and the presence of the white should add black and make them appear darker.”

In terms of our new concept, the explanation is simple. Black borders around the colored area make the color more intense because the chromatic analyzers adjacent to the colored area (in the black) are not being used and so are fully available for use by the stimulated area. On the other hand, if the colored area is surrounded by white, which contains all of the basic colors, the chromatic analyzers which normally serve the colored area are being partially utilized by the white surround, leaving fewer available for use by the colored stimulus so that the latter appears less saturated.

This explanation of the von Bezold spreading effect also explains the experiment in which it was demonstrated that chromaticity is maintained longer on a black background when visual angles are progressively decreased and also the experiment demonstrating the protective effect of a black or white rim surrounding the area in which color is to be induced.

In explaining the phenomena of after

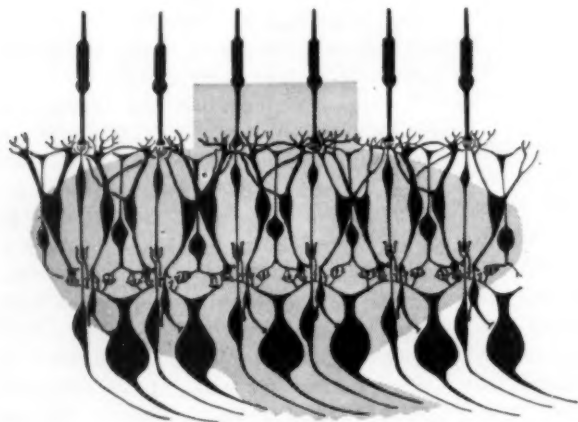


Fig. 2 (Flocks). In this schematic diagram the diffuse gray area indicating a light stimulus is stimulating a small area of the retina (here depicted as two cones), but is utilizing chromatic analyzers (here depicted as being the bipolar cells) which also serve the adjacent photoreceptors (the two cones on each side which have not been stimulated by the light).

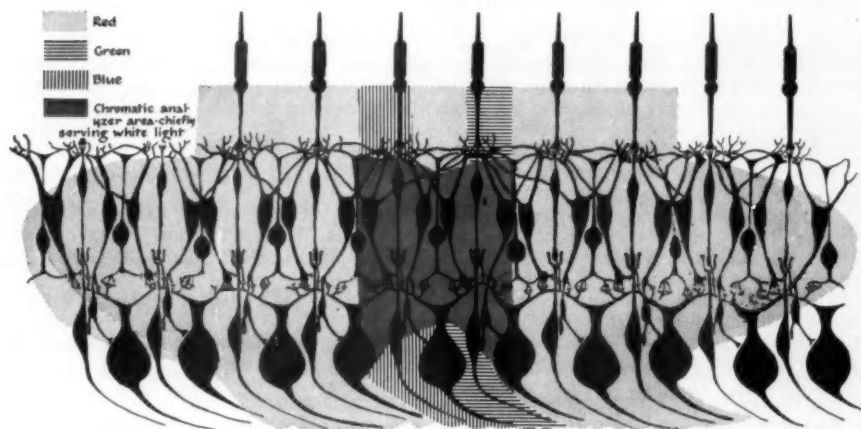


Fig. 3 (Flocks). In this schematic diagram proceeding from the left side, the first two cones have been stimulated by red light, the next two cones by white light (here separated into its components, red, green, and blue), the fifth and sixth cones have been stimulated by red light and the seventh and eighth cones have not been stimulated. (These small areas of retina are depicted as only having two cones to simplify the diagram. The stimuli used in the experiment encompasses areas of retina which contain thousands of cones.) The chromatic analyzers for red light which normally serve the third and fourth cones of this diagram are being partially utilized to analyze the red light stimulating cones 1, 2 and cones 5 and 6 so that they are not available for use by cones 3 and 4 which are being stimulated by green, blue and red light. Consequently the white light striking this area produces a blue-green response. For simplicity's sake the chromatic analyzer area chiefly serving the cones being stimulated by white light is not depicted as spreading laterally to serve the adjacent area also.

images, one can picture the chromatic analyzers (or bipolar cells if that is what they are) continuing to vibrate after the stimulation relayed to it from the photoreceptors had stopped. If the gaze is then transferred to a dark field, the message relayed from the still recovering chromatic analyzers causes an after image to appear in the original colors, a positive after image. If instead the gaze is transferred to a white field, the still recovering chromatic analyzers are relatively refractory to the new white stimulus and so the after image is complementary in color. If one postulates that the rate of recovery of the different types of chromatic analyzers (or bipolar cells) is different, then as the after image fades, one type after another of chromatic analyzer will dominate the response and the after image will appear in a succession of different colors.

SUMMARY AND CONCLUSIONS

The contrast phenomena may be divided

into successive contrast and simultaneous contrast. Evidence is presented which has led to a new concept of the physiologic basis of simultaneous color contrast. This concept is based on evidence that the chromatic analyzers which serve the photoreceptors of a particular portion of the retina also serve the photoreceptors of the immediately adjacent retina. When adjacent areas of the retina are stimulated by lights of different colors, chromatic analyzers being utilized by the photoreceptors of one area of the retina are unavailable for use by the photoreceptors of the adjacent area.

By keeping the subject of color contrast out of the realm of the psychological and making the phenomena predictable and readily understood, this new concept should be of considerable value to artists, photographers, and other people working with color.

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STUDIES OF THE ANTIGENIC PROPERTIES OF ALPHA CHYMOTRYPSIN

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The discovery and development, by Barraquer, of enzymatic zonulysis, using alpha chymotrypsin, has been studied and confirmed by several investigators.¹⁻⁶ These reports demonstrate that this enzyme facilitates the intracapsular extraction of the lens with minimal deleterious effects on the intraocular tissues.

During the clinical investigation of alpha chymotrypsin in enzymatic zonulysis, inquiry was made of the possible allergenicity of this enzyme. Of the 211 cases studied in this clinical investigation, no indication of allergic reactivity was observed and none have been reported in the literature covering enzymatic zonulysis by alpha chymotrypsin.

Chymotrypsin is a proteolytic enzyme, first demonstrated by Vernon,⁷ and isolated from beef pancreas in three crystalline forms (α , β , and γ -chymotrypsin), by Kunitz and Northrop.^{8,9} The three forms differ in molecular weight, solubility, crystalline structure, and rate of inactivation. Alpha chymotrypsin has been shown to be more active and to have a higher molecular weight (41,000) than the other two forms. It occurs in nature in the form of its inactive precursor, chymotrypsinogen. The addition of trypsin initiates the conversion of chymotrypsinogen to chymotrypsin. Alpha chymotrypsin acts only on the peptide linkage which are not close to free amino or carboxyl groups. It has therefore been classified as an endopeptidase.

This study reports the antigenic effects of alpha chymotrypsin as observed in rabbits and guinea pigs.

* From the Research Laboratory, Alcon Laboratories, Inc. Presented at the MidSouthern Section Meeting of the Association for Research in Ophthalmology at Houston, Texas, November, 1959.

METHODS AND MATERIALS

The alpha chymotrypsin used in these studies was a purified crystalline product currently commercially available for professional use in enzymatic zonulysis. Fresh solutions of this enzyme were prepared for each procedure in a balanced salt diluent.

Albino rabbits and guinea pigs were employed as experimental animals. The average weight of the rabbits was 2.0 kg. and the guinea pigs' average weight was 250 gm. The animals were quarantined for two weeks prior to use and were fed a well-balanced diet of pellets and fresh vegetables. The animals had no history of prior exposure to the test materials.

Three procedures were designed to test the antigenicity of the enzyme:

I. *Local organ sensitization.* All rabbits were sedated with nembutal and one eye was anesthetized topically by the application of two drops of 1.0 percent solution of tetracaine. The opposite eye remained untreated to serve as a control. Seven animals were injected intraocularly (anterior chamber), with a 26-gauge needle, with 75 proteolytic units of alpha chymotrypsin solution contained in 0.1 ml of balanced salt solution. After a period of 17 days each rabbit received an intravenous injection (marginal ear vein) of 5.0 ml of alpha chymotrypsin solution containing 1,140 proteolytic units per ml. One rabbit was injected intraocularly with 0.1 ml of equine serum. After 17 days, this rabbit received an intravenous injection of 5.0 ml of equine serum into the marginal ear vein.

II. *Systemic sensitization.* Eight guinea pigs were administered three intraperitoneal injections of 0.2 ml of alpha chymotrypsin solution (150 units per injection) during a six-day period. Four guinea pigs received three intraperitoneal injections of 0.2 ml of equine serum during the same period, to serve as positive control animals. Four guinea pigs remained uninjected to serve as negative controls. After a period of 17 days following the last injection, the four alpha

chymotrypsin animals and the four uninjected animals were challenged by the intravenous injection of 2.0 ml of alpha chymotrypsin solution containing 275 units per ml. The four equine serum animals were challenged by the intravenous injection of 2.0 ml of equine serum.

III. *Precipitating antibody production.* Two rabbits were given separate intraperitoneal injections of 0.5 ml. (275 units/ml) of alpha chymotrypsin and 0.5 ml of equine serum. A third animal received only intraperitoneal injection of 0.5 ml (275 units/ml) of alpha chymotrypsin. A fourth animal received only intraperitoneal injections of 0.5 ml equine serum. These injections were given daily, with two-day intervals every five days, for a total period of 20 days. On the 21st day, the day after the last injections, each animal was bled through the ear vein and the serum was collected. A determination was made of the titer of each product used as the antigen. A concentration of 350 units of alpha chymotrypsin was used for the test.

RESULTS

None of the seven rabbits injected with alpha chymotrypsin intraocularly and challenged intravenously developed any symptoms indicative of a hypersensitivity reaction during the 24-hour period of observation. The animal receiving the intravenous injection of equine serum developed a mild iritis and aqueous flare in the previously injected eye which disappeared within three hours. All animals were subjected to slitlamp microscopy repeatedly over a 24-hour period following the intravenous challenge.

The seven guinea pigs challenged with intravenous administration of alpha chymotrypsin failed to exhibit any signs of hypersensitivity. One animal died within five minutes after challenge but on autopsy no pathologic signs of anaphylactic shock were demonstrable. All four guinea pigs challenged with equine serum died of acute anaphylactic shock within 10 minutes after intravenous injection. All four guinea pigs which served

as controls and received only the challenge injection of alpha chymotrypsin showed no symptoms of hypersensitivity and remained normal throughout a 24-hour period, at which time observation was discontinued.

The two rabbits receiving the combined course of immunization with equine serum and alpha chymotrypsin produced a precipitin titer of 1:16,000 and 1:8,000 against equine serum and no detectable titer against alpha chymotrypsin. The one rabbit which had been immunized with equine serum alone produced a titer of 1:16,000, and the animal injected with alpha chymotrypsin alone failed to produce a detectable precipitin titer.

DISCUSSION

According to Martin, the majority of studies on the antigenicity of enzymes has been carried out with impure preparations and can be regarded as having little bearing on the problem of the administration of highly purified crystalline enzymes.¹⁰ The enzymes, however, are basically protein molecules and as such should possess antigenic properties. Starin, however, has reported that gelatin, a protein, was nonantigenic.¹¹

The amount of antigen necessary to elicit antibody formation is relatively small. Rose-nau and Anderson¹² found that 10^{-6} ml. of equine serum (7×10^{-8} gm. protein) would sensitize a guinea pig. According to Boyd,¹³ one subcutaneous injection of one mg. of equine serum is sufficient to sensitize a guinea pig for an anaphylactic shock.

In the series of tests reported here, the animals received dosages of alpha chymotrypsin comparable to the average dose administered to the human eye during enzymatic zonulolysis. Since the guinea pigs averaged 250 gm., the equivalent dosage computed on a weight basis would be 280 times that normally administered to the human eye for potential absorption.

Slepian reported that antibodies have not been demonstrated in patients, following the intravenous administration of trypsin. Chymotrypsin and trypsin have been available for several years. Of the thousands of cases treated, only two have been reported in which reactions resembling anaphylactic shock were noted.¹⁴

CONCLUSION

In the series of tests employed in the evaluation of the antigenicity of the enzyme alpha chymotrypsin it has been demonstrated that:

1. The intraocular injection of alpha chymotrypsin failed to induce either local ocular or systemic sensitization of rabbits to intravenous challenge with the enzyme.

2. Intravenous challenge with equine serum of one control rabbit sensitized by the intraocular injection of equine serum evoked a mild iritis and aqueous flare in the injected eye.

3. Intravenous challenge with alpha chymotrypsin in guinea pigs previously subjected to sensitizing injections of the enzyme failed to elicit signs or symptoms of anaphylactic shock.

Control animals sensitized and challenged with equine serum succumbed to typical fatal anaphylactic shock.

4. Attempts to elicit precipitating antibodies by a prolonged series of intraperitoneal injections in rabbits failed to produce a detectable titer for alpha chymotrypsin, as determined by the ring precipitin test. High titers of precipitating antibodies were produced in response to concurrent immunization with equine serum.

With the quantities of the proteolytic enzyme, alpha chymotrypsin, employed in these procedures, no antigenic response was detected in experimental animals.

P.O. Box 1959 (1).

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VISUALIZATION OF WAVE-GUIDE MODES IN RETINAL RECEPTORS

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INTRODUCTION

In this study wave-guide modes have been visualized as emitted from receptor outer segments in the retinas of albino rats, of rhesus macaque monkeys, and of humans. Therefore it may be hypothesized that this is the normal form of energy transfer in the retinal receptor outer segments of these species.

A wave-guide literally channels electromagnetic waves along a given path. In essence, the complex phenomena discussed in this article are the result of interactions which occur in wave-guides. These distributions of energy (wave-guide modes) may be described mathematically by Bessel or Hankel functions which may be identified in the

shorthand notation employed in reference to each mode. A wave-guide modal pattern may be defined as a distribution of field which propagates down a wave-guide with a well defined phase velocity.

It is assumed by most investigators today that the retinal receptors act as dielectric wave-guides. This assumption is based upon our knowledge of the physical properties, and the configuration of the retinal receptors. Jean and O'Brien,¹ in an experiment in which they employed model retinal elements magnified into the micro-wave spectrum, and Toraldo,² in theoretical discussion, established the concept that the retinal receptors act as dielectric wave-guides. Ronchi and Toraldo have presented a study³ of the scattering properties of the retina, the results of which were in keeping with theoretical arguments. Wald⁴ may have photographed very complex wave-guide modal patterns in the large diameter retinal rods of the frog (*rana pipiens*). These were not recognized as such by that author in the very fascinating discussion accompanying this photograph.

* From the Department of Ophthalmology, Washington University School of Medicine and the Oscar Johnson Institute. This research has been supported in part by Grant B-2168 from the National Institute of Neurological Diseases and Blindness; National Institutes of Health, Public Health Service, Bethesda, Maryland. Submitted for publication November 10, 1960 and read before the Mid-winter National Meeting of the Association for Research, New Orleans, Louisiana, December 6, 1960.

ELEMENT II

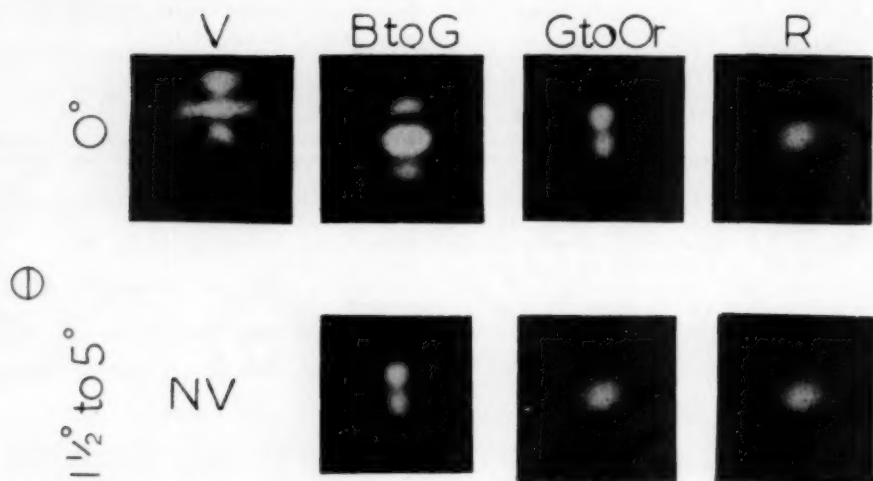


Fig. 1 (Enoch). Modal patterns photographed in a glass fiber. V = violet, B = blue, G = green, Or = orange, R = red, NV = not visible, θ = angle of obliquity of incidence of the chief ray at the fiber. (Courtesy National Physical Laboratory (Photographic Section), Teddington, Middlesex).

Snitzer, Osterberg, and their co-workers recently demonstrated that wave-guide modes could be visualized in the visual spectrum in micron-class glass fibers.^{5,6} Kapany and his group also have studied related aspects more recently.⁷ Retinal receptors have dimensions and physical properties not too very different from Dr. Snitzer's fibers. In this paper, this author has sought to clearly demonstrate the presence of wave-guide modes in retinal receptors. In addition to firmly establishing the fact that the receptors act as dielectric wave-guides, interest is directed toward determining which modal patterns are seen and under what conditions. The latter phase of the problem is only introduced in this paper. This author has previously introduced discussion as to the possible role (roles) of the modal patterns in the visual act.⁸

APPARATUS AND PROCEDURE

Through the kindness of Dr. Elias Snitzer of the American Optical Company, the au-

thor was able to spend considerable time studying wave-guide modes produced in glass fibers. The fibers Dr. Snitzer provided had physical properties such that they approached those of the retina as closely as possible within the limitations of that material.

The following properties of the mode form of transmission are of considerable interest:

1. Mode transmission gives rise to non-uniform distributions of energy in the wave-guide.
2. The mode transmitted varies as a function of the wavelength of the radiant energy employed. Ordinarily two wavelengths produce two superimposed patterns.⁹
3. The modal pattern varies as a function of the angle of incidence of the radiant energy. In most instances the modal pattern which appears when the dielectric wave-guide is irradiated obliquely (with a given wavelength) is the same

as that produced when the light is incident normally and the wavelength increased.

4. In a given fiber different modal patterns appear to vary in the intensity of their excitation and/or their transmissivity (essentially a filter effect varying with wavelength). Obliquity of incidence of the radiant energy also influences the result.
5. The larger the diameter of the fiber relative to the wavelength of the radiant energy employed the more patterns may be propagated. Phrased in another sense, one may say that the shorter the wavelength of the radiant energy the more patterns may be propagated. This in no way states which of the patterns (which may be propagated) is (are) propagated.

Figure 1 is a presentation of modal patterns photographed as they are emitted from one of Dr. Snitzer's fibers. The diameter of this fiber was slightly larger than one micron. The index of refraction of the fiber (core) was 1.56 and the index of the surrounding media (cladding) was 1.52. It will be noted that this is a single fiber having a length of a little less than one inch. In this case one is not faced with the possibility of the excitation in one fiber influencing events in a neighboring fiber. This type of interaction between fibers presents separate problems which are briefly discussed below. Some of Dr. Snitzer's fibers were ground down to lengths corresponding to those of retinal receptors. Mode transmission was still evident. The apparatus employed was essentially identical with that described below. In Figure 1, note the modal patterns seen, the types and number of mode changes which occur, and the order of magnitude of the angles of obliquity involved. The pattern seen to the side of the mode observed in the violet is an artifact.

In order to obtain a positive result when searching for specific wave-guide modes in

retinal receptors, certain limitations must be imposed. Since modal pattern changes with wavelength, it becomes necessary to use a source of narrow band monochromatic energy in order to isolate given modes or modal patterns. Further, because modal pattern varies with angle of incidence, it becomes necessary to use only a relatively small cone of incident energy. In the apparatus employed, the cone of light subtended approximately two degrees. It is important to remember that the incident cone of energy at the retina subtends only a rather small solid angle except under conditions of maximal pupil dilation (1 mm in the entrance pupil = 2.5° at the retina). Thirdly, it becomes necessary to properly orient the retinal receptors in relation to the total optical system. In addition, problems of post-mortem changes, tonicity of accessory solutions, etc., must be considered.

The apparatus employed involved various powerful sources of energy. Ultimately an Osram XBO 1001 Xenon arc was employed (lower left hand corner of Figure 2). The light was first passed through a water chamber. The arc was imaged upon the entrance slit of a Gaertner Spectrometer modified to serve as a monochromator by means of the introduction of a second slit. The exit slit of the monochromator could be imaged normally upon the excised retinal tissue. Some variation in angle of incidence was possible. An aperture placed near the lens imaging the exit slit of the monochromator provided control of the size of the cone of light incident at the specimen. This tissue was viewed using an oil immersion objective and photographed with a Leitz Aristophot unit, Ansco Super Hypan film, Kodak Royal X-Pan film, and Polaroid "3000" film were used on different occasions. With all films time exposures were required, and in the case of the former two films, extended development periods were often employed. Because of the inherent graininess of these films maximum magnification possible was obtained in the original exposure.



Fig. 2 (Enoch). Photograph of the apparatus. The main elements are light source, water chamber filter, lens focusing the source on the entrance slit of the monochromator, monochromator, lens focusing the exit slit of the monochromator on the specimen, the specimen, microscope, and photomicrography unit.

The main procedure employed in preparation of the specimen was one shown to the author by Dr. Katherine Tansley¹⁰ of the Institute of Ophthalmology, London. The enucleated eye was placed in normal saline and all tissue was dissected away from the retinal cup. A piece of the retina was removed and was placed in a recessed chamber (in normal saline) in one of a number of specially prepared cover slips (courtesy, National Physical Laboratory, Teddington, Middlesex). The depth of the recessed chamber employed corresponded to the thickness of the retina. A microscope slide was placed above the chamber holding the sample. The unit was inverted, and was then placed on the microscope stage. The ends of the outer segments of the receptors were placed nearest to the microscope objective and the incident radiant energy travelled through the speci-

men as it might have passed through the retina *in situ*.

Various concentrations of saline were employed in order to determine whether small variations in this factor significantly affected results. A chamber into which fluid could be introduced and withdrawn was constructed to test this phase of the problem. It was found that this factor was not a significant source of error. If one markedly changed the salinity, for example, employed a highly hypotonic solution, one could change the physical properties of the receptors and interstitial fluid. When this was done, the patterns photographed were markedly changed, and the cells distorted. Occurrences of this type obviously will have analogy in certain pathologic events. The significance of such variations in physical properties depends on many factors, only one of which is the possible role played by wave-guide modes in vision.

RESULTS

In order to establish the fact that these distributions were truly wave-guide modes, it became necessary to demonstrate that a single receptor changes modal pattern under one of the conditions noted when one of Dr. Snitzer's fibers was viewed. Verification was most easily provided by varying wavelength rather than obliquity. Examples of this type of finding are shown in Figure 3 for each of the three species. These were obtained by overlaying photomicrographs of the same preparations, taken at two different wavelengths.

The quality of these presentations is frankly less than might be desired. It is evident that in future work techniques must be refined in order to provide improved renditions of those features which are so evident through the microscope.

In this first study, no concentrated effort was made to identify rods and cones as separate entities in the plane of the receptor outer segments. The modal patterns seen are readily recognizable as the ones seen in Dr.

MODAL PATTERN CHANGES INDUCED BY VARYING WAVELENGTH

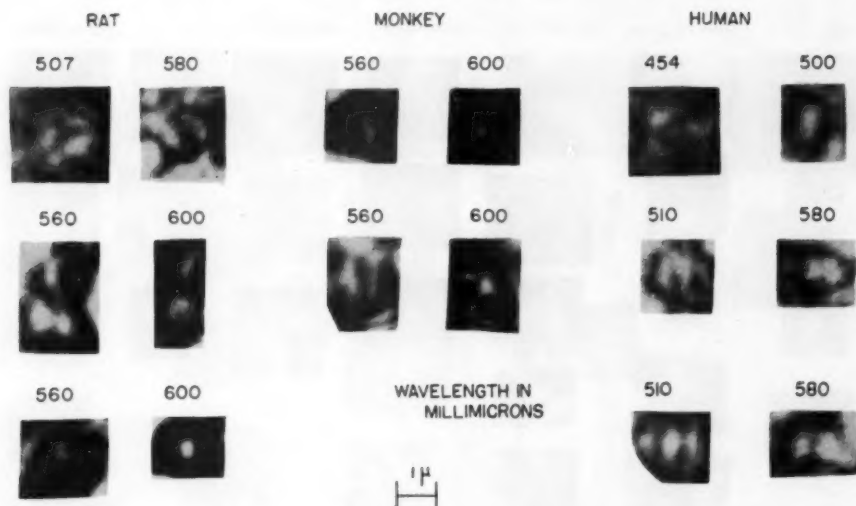


Fig. 3 (Enoch). Changes in wave-guide modal patterns observed with wavelength variation in the three species tested.

Snitzer's fibers. *At all times modal patterns have been seen in all receptors* in the three species investigated. Plane of focus is very delicate for any single receptor. Since the receptors do not terminate uniformly, the main emitted pattern is not seen in exactly the same plane in all receptors. Hence, it is difficult to photograph a mosaic. The radiating nature of these patterns is observed by racking the microscope upward. If one racks the microscope downward from the point at which a given modal pattern is first focused, the luminance of a given pattern varies (usually decreases) as it is not radiated out of the receptor in any sense uniformly along its length. In some instances the modal pattern changes with this operation. In some instances this change is associated with change in diameter (cones). In other instances one cannot say whether this indicates differences in physical properties in the receptor, or mode interactions.

In general there is a predominant modal pattern emitted from the outer segments in a given retinal area. Whether departures

from this dominant modal pattern represent cones versus rods, or local physical differences, or interaction differences, was not assessed at this time. It is evident, however, that outer segments of receptors which vary in *diameter*, *obliquity* (orientation), or cross-section often present different modal patterns in a given photograph.

It can be stated that the number of modes transmitted by the receptors when wavelength is varied in the visible spectrum is not large. The author does not remember seeing more than two mode changes in a single receptor outer segment when varying wavelength from about 450 m μ to 600 m μ . In a large proportion of the receptors the modal pattern does not seem to change at all. Whether this is in fact the case in these receptors, or whether they are oriented somewhat obliquely and are not as susceptible to mode change cannot be answered.

Thus, one can say that the mode form of transfer of energy is probably the normal manner in which light passes down the receptor outer segments in the rat, rhesus

RAT WAVE-GUIDE MODAL PATTERNS

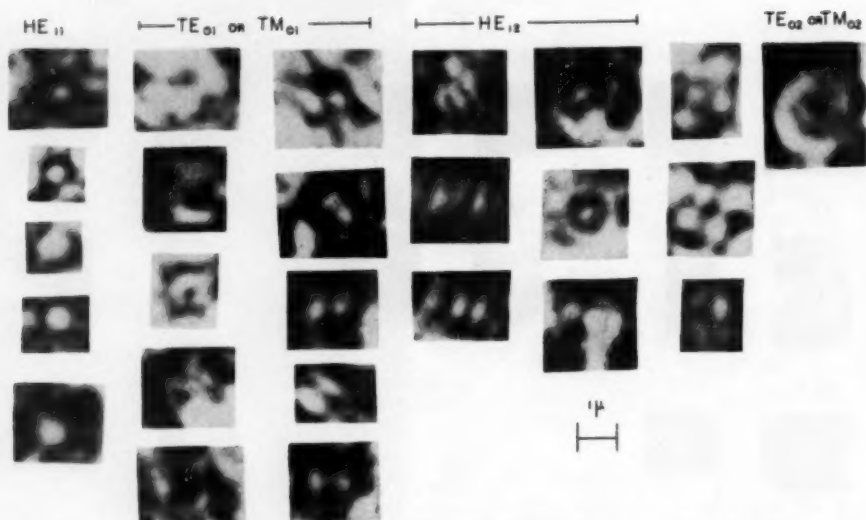


Fig. 4 (Enoch). Wave-guide modal patterns seen in the receptor outer segments of rats. The most commonly seen modes are those to the left of the figure.

macaque monkey, and man. Figures 4, 5, and 6 present modal patterns frequently seen in each of the three species.* In each instance, the nomenclature employed implies which distribution each modal pattern resembles. In some, there may be interactive effects present, and in others, microvibrations may have caused distortions. Since the vast bulk of the data obtained to date have been on rat eyes, one sees a larger number of presentations in Figure 4. Modal patterns other than the ones presented are occasionally seen. Of these, some may be interactive forms. A rat retinal mosaic is seen in Figure 7. The HE_{11} and TE_{01} (or TM_{01}) modal patterns (fig. 8)* are most frequently seen. These modal patterns will be discussed at greater length below.

Histological support is available to show that the receptors are intact in these type preparations, and that much of the ground

substance (located about the receptors) remains.

The eyes of all animals employed were normal to the best of the knowledge of the investigator. Since one might question the role of the post-mortem changes, in some instances preparations were put into position for viewing within five minutes after death.¹² Viewing a given pattern in such a specimen during the normal experimental period (about one hour) resulted in no visible change in the modal patterns.

The first of the two human eyes obtained manifested absolute glaucoma, with lens and corneal opacities, and some evidence of retinitis proliferans (on the side of the retina opposite to the one employed). The second human eye obtained revealed evidence of Fuch's dystrophy, had had a prior cataract operation, a corneal transplant which had opacified, and an uncontrolled glaucoma. Hand motion vision was present prior to surgery, and postoperative dissection of the eye revealed liquified vitreous,

* Recent computations show that some modifications are necessary. Please refer to the addendum to this paper.

MONKEY WAVE-GUIDE MODAL PATTERNS

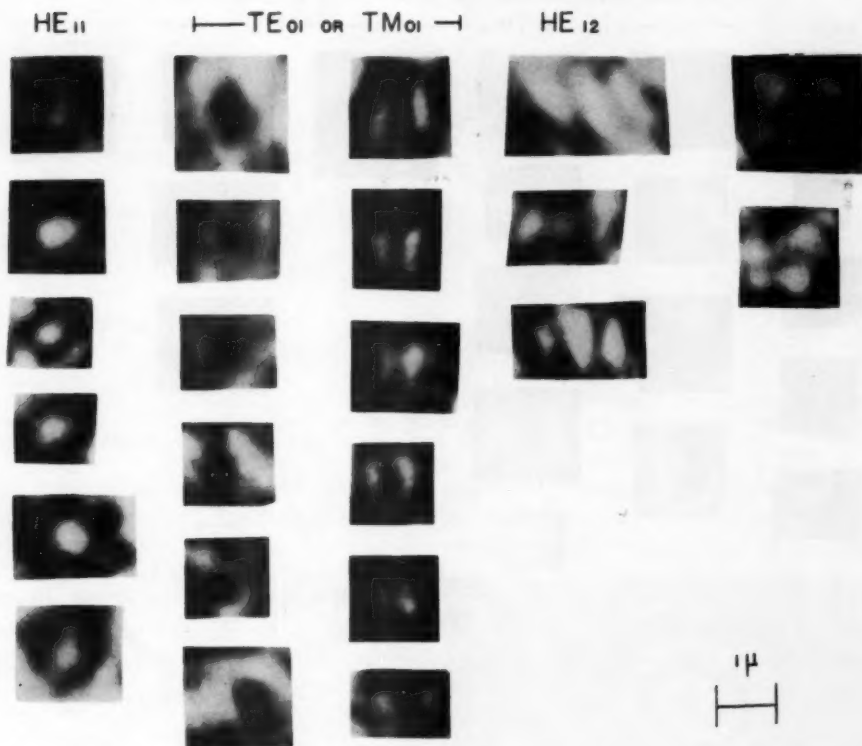


Fig. 5 (Enoch). Wave-guide modal patterns seen in the receptor outer segments of rhesus macaque monkeys. The most commonly seen modes are those to the left of the figure.

and some retinal detachment. Dissection was initiated within ten minutes of enucleation in both instances. Obviously, the dissection technique had to be varied somewhat here, but in general, the principle was the same.

DISCUSSION

Having established the fact that the mode form of energy transfer is present, and that mode changes occur in some receptors with wavelength in the above figures it becomes necessary to ask if these phenomena have any effect on vision. At a minimum, differences in energy distribution, and apparent differences in excitation and/or transmis-

sivity of different modes in a given receptor (marked in some receptors) will to an (as yet undetermined) extent modify the visual response. At a maximum, since a spatial physical coding of wavelength information may well exist in that part of the visual receptor associated with photo-reception, it now becomes important to determine if this physical code is translated in some manner into neurological excitation. That is, is this form of coding the first step in our color vision process, or are the differences in transmissivity important from this point of view? This is a most important question, and effort will be directed toward its answer.

HUMAN WAVE-GUIDE MODAL PATTERNS

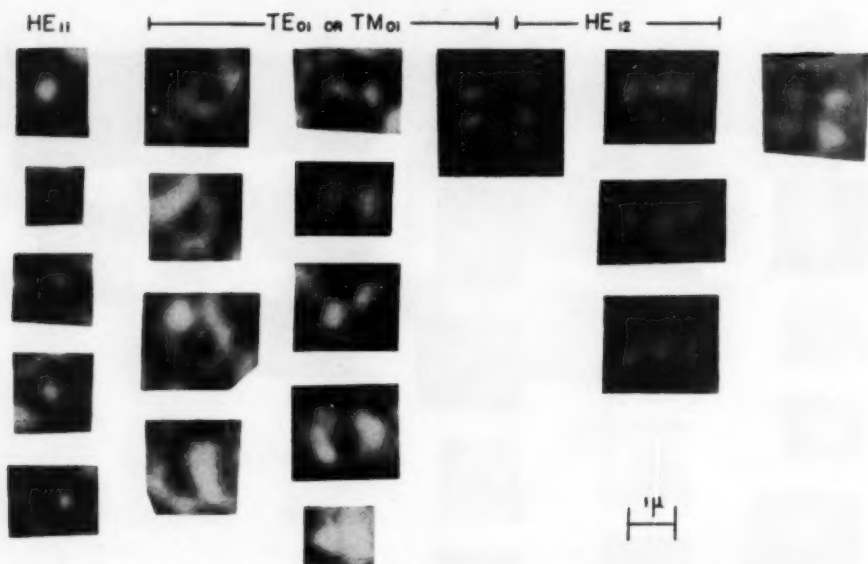


Fig. 6 (Enoch). Wave-guide modal patterns seen in the receptor outer segments of humans. The most commonly seen modes are those to the left of the figure.

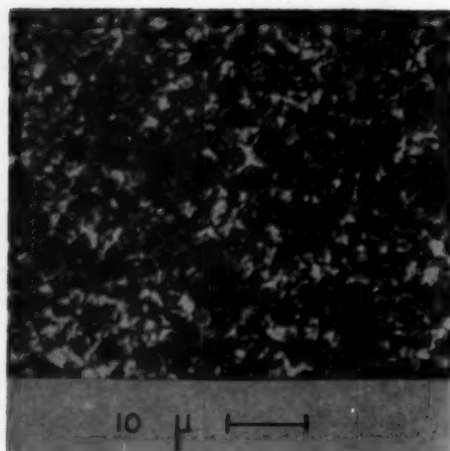


Fig. 7 (Enoch). Rat retinal (outer segments) mosaic showing modal patterns.

MODAL PATTERNS

MODE	NEGATIVE	FORM(S)	CUTOFF
HE ₁₁	•		0.0
TE ₀₁ or TM ₀₁	○	⊖	2.405
HE ₁₂	⊙	⊖	3.832
	⋮		
TE ₀₂ or TM ₀₂	⊙		5.520

Fig. 8 (Enoch). Modal patterns and their associated cutoff values. The forms of the modal patterns presented are schematic.

TABLE 1
DATA ON PHYSICAL CONSTANTS OF THE RECEPTOR OUTER SEGMENTS

A. Index of Refraction:		
Rod	Rat	Monkey
Cone	1.4076 (S)	1.4076 (S)
Interstitial Substance	—	<1.3985 (S)
	1.334–1.347 (B)	1.334–1.347 (B)
B. Diameter of Rod Outer Segments		
Rat	1.4 ± 0.2 μ (S)	
Monkey		
Peripheral	1.8 ± 0.3 μ (S)	
Central	1.3 ± 0.2 μ (S)	
C. Length of Rod Outer Segments		
Rat	13 ± 1.0 μ (S)	
Monkey		
Peripheral	21 ± 1.0 μ (S)	
Central	17 ± 1.8 μ (S)	

Source: (S) Sidman, (B) Barer.

Human index of refraction data are not available. The diameters of human retinal receptor outer segments are of the same order of magnitude as these, but the lengths of the outer segments are longer. Reference: Polyak, C. L., *The Retina*, University of Chicago Press, Chicago, 1941.

What little evidence there is in answer to it has been reviewed in the recently published discussion.⁸

Each given modal pattern, according to Snitzer,¹¹ has what is known as a cutoff parameter which is determined by the physical properties of the receptor and the wave-

length of the electro-magnetic disturbance. A given dielectric wave-guide has an equivalent cutoff parameter which may be computed.

TABLE 2
COMPUTATION OF CUTOFF PARAMETERS

$$A. \text{ Cutoff Parameter} = \frac{2\pi r}{\lambda} \sqrt{n_1^2 - n_2^2},$$

where $n_1 > n_2$, and λ expressed in μ .

$$B. \text{ If } r = 1.4/2 \quad \mu = 0.7 \mu \quad n_1 = 1.40$$

$$n_2 = 1.34 \quad \text{Cutoff Parameter} = \frac{1.783}{\lambda}$$

λ	Cutoff Parameter
.400 μ	4.46
.500 μ	3.56
.600 μ	2.97
.700 μ	2.55

$$C. \text{ If } \lambda = .5 \mu \quad n_1 = 1.40 \quad n_2 = 1.34$$

$$\text{Cutoff Parameter} = 5.096r$$

Diameter	r	Cutoff Parameter
1.0 μ	0.5 μ	2.55
1.4 μ	0.7 μ	3.56
1.8 μ	0.9 μ	4.59
2.2 μ	1.1 μ	5.61

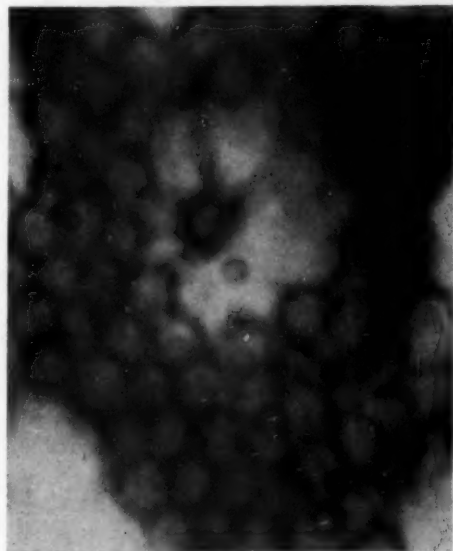


Fig. 9 (Enoch). Photography of a flat preparation showing the presence of a ground substance (possibly an acid mucopolysaccharide) existing between the receptor outer segments in a rhesus macaque monkey. Stained with iron (Rinehart), and hematoxylin. (Courtesy of Dr. T. Yamashita and Dr. F. Fankhauser.)













DESIGNATIONS	NEGATIVE FORM	CUTOFF
HE ₁₁		0.0
TE ₀₁ , TM ₀₁		2.405
HE ₂₁		2.4+
(TE ₀₁ OR TM ₀₁) + HE ₂₁		2.4+
HE ₁₂		3.812
EH ₁₁		3.812
HE ₃₁		3.8+
HE ₁₂ + (HE ₃₁ OR EH ₁₁)		3.8+
HE ₃₁ + EH ₁₁		3.8+
EH ₂₁ OR HE ₄₁		5.2
TE ₀₂ , TM ₀₂ , HE ₂₂		5.52
(TE ₀₂ OR TM ₀₂) + HE ₂₂		5.52

Fig. 10 (Enoch). Schematic presentation of modal patterns having a cutoff of 5.52 or less.

The formula for this cutoff parameter is:

$$\text{Cutoff parameter} = \frac{2\pi r}{\lambda} \sqrt{n_1^2 - n_2^2},$$

where $n_1 > n_2$.

As applied to the retina, in this expression n_1 is the absolute index of refraction of the receptor outer segment, n_2 is the absolute index of refraction of the interstitial fluid, r is the radius of the transverse cross section of the receptor outer segment (or where con-

sideration is given to the ellipsoid or inner segment, the equivalent cross section [and indices] of those parts), and λ is the wavelength of the light employed (in vacuum or air). This ratio is computed using the same units for r and λ .

A given dielectric wave-guide may propagate all modes which have a cutoff parameter which is less than the wave-guide cutoff parameter. This formula does not describe which modal pattern(s) is (are) propagated

if more than one modal pattern may be propagated. Obliquity is not considered in this ratio.

On the basis of Sidman's data,¹² and Barer's analysis,¹³ shown in Table 1, one can compute probable cutoff parameters for different wavelengths and receptor diameters (Table 2). Modal patterns having cutoff parameters of this general magnitude are shown schematically (in negative form) in Figure 8 with their appropriate cutoff parameters. Given modal patterns may appear in somewhat different forms in certain conditions. The alternative forms given in two instances (right side) represent two interactive forms. These patterns may be compared with the photographs shown in Figures 1, and 3 to 6. It is obvious that the patterns predicted are the ones predominantly seen. This in turn offers support to Sidman's data.

One may introduce consideration of problems relating to the influence of electro-magnetic disturbance in one receptor acting in neighboring receptors. The magnitude of such effects depends largely upon the separation of the individual receptor components (particularly the outer segments) and the length of these components.^{18,11} It has long been known that fibrils project from the pigment epithelium layer. These act to stabilize the receptors as to position, to separate the receptors, and no doubt play an important roll in their metabolism. In addition, it is known that a ground substance which may be an acid mucopolysaccharide is also located in the interstices between the receptors (fig. 9). The darker areas stain blue, the lighter areas stain red (in the original) when the stains listed with the figure caption are employed. A recent presentation of receptor spacing is one by Cohen using electron microscopy.¹⁴ On the basis of a paper completed a year ago by this author using larger fibers¹⁶ (supported by a recent study by Campbell and Gregory¹⁷), on the basis of Professor Cohen's photograph and Figure 9, on taking into consideration data regarding receptor dimensions, on the basis of resolu-

tion data determined by interferometric techniques,¹⁸ and on the basis of theory,^{18,11} it can be inferred that in *normal* retinas that this problem is not too great.

SUMMARY AND CONCLUSION

Distributions of energy emerging after passing through the retinal receptors have been photographed. The conditions of retinal irradiation and receptor orientation were identical with those which might be found in the eye. It has been possible to demonstrate that the normal form of energy transfer in retinal receptors is similar to that found in dielectric wave-guides. Modal pattern changes have been recorded with variation in wavelength of the incident energy. Examples of modes seen in rat, monkey, and human retinas have been presented.

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The author wishes to acknowledge the kind assistance and advice of Drs. Elias Snitzer and Franz Fankhauser in many phases of this study, and to praise the work of his assistant Mrs. Verena Fankhauser.

Many other individuals, too numerous to mention have offered advice on various problems encountered in this work. To them, the author wishes to express his appreciation.

ADDENDUM

Recent computations of modal patterns having a cutoff of 5.52 or less by Dr. Elias Snitzer of the American Optical Company show that the earlier assignment of modal pattern designations needs some modification. Dr. Snitzer's new computations will appear in an article in the April, 1961 issue of the *Journal of the Optical Society of America*.

The accompanying figure (fig. 10) provides a list of the modal patterns. Some of the resultant patterns are the result of combinations of the listed designated distributions. The patterns having a cutoff greater than 5.0 are seen extremely rarely when focusing upon receptor outer segments. All have been seen, but not all have been recorded on film. Further differentiation of these modal patterns is possible by means of inserting a polarizer in front of the receptor plane, and

an analyzer in the microscope. A single polarizer in front of the receptor has virtually no effect upon patterns observed when viewing through the microscope.

Recently, normal freshly obtained human retinae have been available. All conclusions presented in this article based upon human eyes exhibiting pathology prove to be valid.

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SOME MEMBRANE PROPERTIES OF NON-PIGMENTED CILIARY EPITHELIUM OF POIKYLOTHERMIC VERTEBRATES

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Membranes of living cells probably do not behave like inert membranes. However, in some respects living membranes seem to re-

spond to external potassium ion concentrations according to the Nernst equation,

$$E = -\frac{RT}{F} \log \frac{[K^+]_{int}}{[K^+]_{ext}} \quad (1)$$

where E is the potential across the membrane; R, T, and F have their usual significance; and internal and external potassium ion concentrations are in the log ratio.

This equation predicts the behavior of

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membranes of nerve fibers (Hodgkin & Huxley³) but not of muscle cells (Shaw, Simon and Johnstone⁶). The discrepancies were reconciled by Shaw et al by assuming that only part of the internal potassium is concerned in regulation of membrane potential. On the other hand, cell membrane potential has not appeared to respond to temperature as predicted by the Nernst equation (Hodgkin & Katz, nerve fibers⁴), (Apter and Koketsu, muscle cells²), (Apter,¹ nerve cells). In addition, nerve cell membrane resistance responds to external potassium ion concentrations before the potential responds (Apter), refuting applicability of the Nernst equation still more strongly.



Fig. 1 (Apter). Diagram of specimen from eye. A is iris, B is choroid, C is non-pigmented ciliary epithelium, D is retina.

The present study applies this last test to the non-pigmented ciliary epithelium of bullfrog, toad and turtle eyes. This cell type was chosen because it was readily accessible for impaling with a micropipette and was durable, though impaled. Even more important, it is a secreting cell, the membrane properties of which have not yet been investigated with methods found reliable for mus-

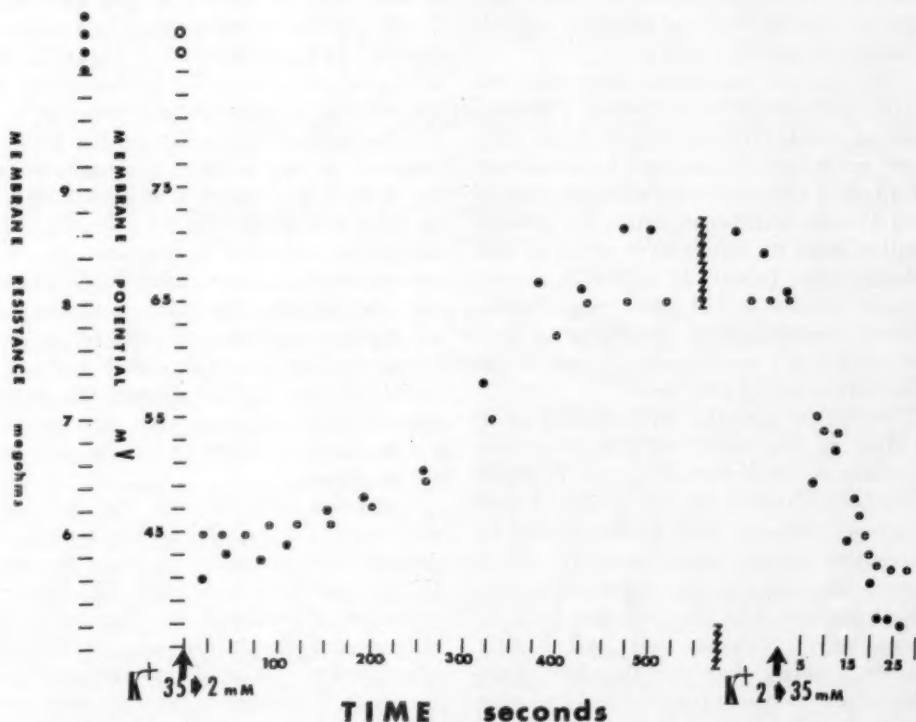


Fig. 2 (Apter). Time course of the response of a single cell from the frog ciliary epithelium to an abrupt change in $[K^+]_{ext}$. The resistance (●) and potential (○) rose simultaneously when $[K^+]_{ext}$ was changed from 35 to 2 mM/liter. In terms of this time scale, the concentration change was practically instantaneous, but the response was slow. The membrane properties dropped precipitously when $[K^+]_{ext}$ rose from 2 to 35 mM/liter, resistance dropping first.

cle and nerve cells. The present findings, therefore, test the applicability of the Nernst equation and find it not tenable; support an alternative explanation for control of potential by the potassium ion; and elucidate the behavior of the membrane of one side of a secreting cell.

METHOD

Eyes freshly enucleated from toads, frogs and turtles were incised through the sclera, limbus and cornea along two meridians meeting at the corneal apex and at the optic nerve. The underlying uvea and retina were removed gently in toto with careful section of the zonule fibers. If the lens capsule was cut accidentally, specimens were not suitable because a thick film covered the ciliary epithelium, making external solutions and micropipettes inaccessible to it.

The choroid was peeled away from the retina and reflected under the iris. The junction of retina and choroid at the ciliary body was not disturbed (See fig. 1). In this way the ciliary processes were exposed, projecting over the transparent retina. Transillumination made the single layer of ciliary epithelial cells (about $25 \times 35 \times 25$ micra) readily visible at 120 times magnification. Under visual control micropipettes constructed with a sharp taper impaled the outside membrane of single cells.

Membrane potential was recorded on an Offner recorder monitored with a Tektronix oscilloscope. Both instruments had negligible drift of DC potentials. By running a small current (10^{-9} amp) through the impaling micropipette at one second intervals for 20 msec, the potential rose exponentially to a level determined by the resistance of the cell membrane. (Koketsu, Cerf, and Nishi⁹). With a single electrode, therefore, membrane resistance and potential could be measured simultaneously.

Specimens were immersed and immobilized in a bath at 15°C continually irrigated from four reservoirs containing graded concentrations of potassium ions. Sodium (112 mM), Calcium (1.8 mM), and tris buffer

to maintain pH at 6.8 were also present. By shifting irrigation from one reservoir to another, the time course of the change in resistance and in potential to abrupt changes of external potassium ion concentrations were registered. What is more, the steady state relationships between $[\text{K}^+]_{\text{ext}}$ and membrane resistance and potential were determined. Experiments were carried out with and without (10^{-8} mgm percent) in the acetazolamide (Diamox) solutions.

RESULTS

Time course of the response of membrane properties to a change in $[\text{K}^+]_{\text{ext}}$. A properly impaled cell maintained a steady resting potential and resistance at any given $[\text{K}^+]_{\text{ext}}$ between 3 and 35 mM for at least one hour. The time course of the response to a sudden shift in $[\text{K}^+]_{\text{ext}}$ is depicted in Figure 2. It illustrates that a rise in $[\text{K}^+]_{\text{ext}}$ brought on a prompt drop in resistance and potential, resistance responding several seconds before potential; a drop in $[\text{K}^+]_{\text{ext}}$ brought on a rise in these parameters, both responding at the same rate which was not prompt at all. All species responded in this way. At concentrations of potassium below 3 mM resistance did not alter detectably, but potential was higher at zero than at 3 mM $[\text{K}^+]_{\text{ext}}$. Although the time course depended on the direction of the $[\text{K}^+]_{\text{ext}}$ change, the levels reached at steady state were reproducible and characteristic of the $[\text{K}^+]_{\text{ext}}$, as the next section shows.

Correlation of resistance and potential with steady $[\text{K}^+]_{\text{ext}}$. The steady state relationship between membrane properties and $[\text{K}^+]_{\text{ext}}$ are depicted in Figure 3. At concentrations above 2 mM, the relationships of resistance and potential seem to parallel each other. However, at low K ion concentrations the potential continues to rise while resistance does not change. A negative sigmoid relationship existed, therefore, between resistance and $[\text{K}^+]_{\text{ext}}$. This relationship fit a normal distribution curve as well as being rectifiable by plotting the log of a ratio of resistance values against the reciprocal of the $[\text{K}^+]_{\text{ext}}$. The pre-

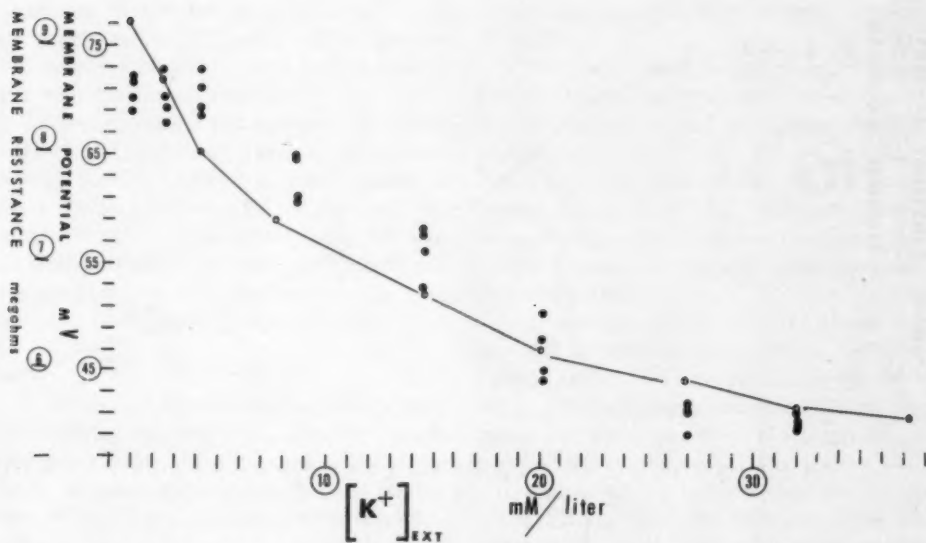


Fig. 3 (Apter). Steady state relationship between potassium ion concentration outside turtle ciliary epithelial cells and the resistance (●) and potential (○) of the exposed membrane of those cells. Each point is the mean obtained for one hundred cells impaled consecutively not always on a single specimen. Cells, animals and specimens could not be shown to differ significantly from one another. The line joins mean values of membrane potential. It did not conform sufficiently well to an exponential or hyperbolic transform to be expressed by any single valued function.

cise form, therefore, was not determined. The potential— $[K^+]_{ext}$ relationship was also not amenable to expression as a single valued function. However, the curve seems to fit an exponential form at concentrations of potassium above 5 mM. The deviation at lower concentrations may result from loss of potassium from the cell (Tobias⁷). Until the extent of loss can be measured, it would be premature to analyze the form of the curve.

Membrane properties in the presence of Diamox. Diamox (10^{-5} mgm percent) altered the relationships of Figure 3 by shifting them to the right (fig. 4). In other words, Diamox made the resistance and potential less sensitive to a wide range of potassium ion outside the cell. Important, too, is the obvious sigmoid relationship for both resistance and potential which could not be shown to differ significantly from one another. These curves fit the form,

$$E = \frac{E_{max}e^{C/[K^+]} + E_0}{1 + e^{C/[K^+]}} \quad (2)$$

where E is the potential (or resistance) at any external potassium ion concentration, $[K^+]$; E_{max} is the potential (or resistance) at lowest $[K^+]$ and E_0 at highest $[K^+]$; C is a constant which probably includes the parameter, temperature.

CONCLUSIONS

These findings indicate that the external membrane of the non-pigmented ciliary epithelium responds to changes in the concentration of potassium in the bathing fluid. These responses are similar to those described for muscle (Apter & Koketsu²), (Shaw, et al.⁶) and nerve cells (Apter¹). The present detailed data indicate that this epithelial cell membrane does not behave like an inert membrane for the following four reasons: (I) Resistance changes by an appreci-

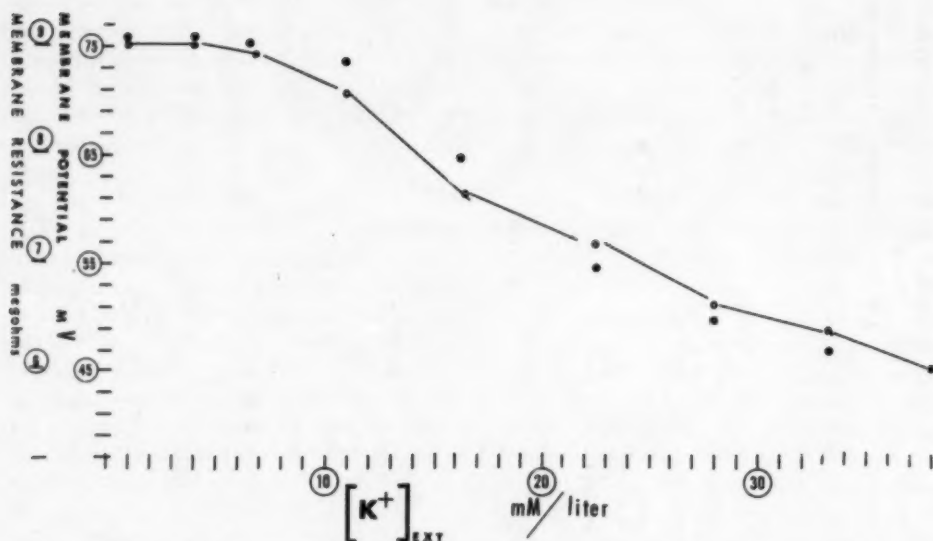


Fig. 4 (Apter). Steady state relationship between external potassium ion concentration and resistance (●) and potential (○) of ciliary epithelial cells, in the presence of diamox (10^{-3} mgm percent). The two sets of data could not be shown to differ significantly from one another. Each point is a mean of 75 determinations made on six turtle eyes.

able increment of time before potential during an abrupt change from one $[K^+]_{ext}$ to another. (II) Resistance and potential change more rapidly when $[K^+]_{ext}$ is raised than when it is lowered. (III) Resistance and potential do not have the same relationship to $[K^+]_{ext}$ below 5 mM potassium concentration. (IV) A carbonic anhydrase inhibitor alters the relationship between membrane properties and $[K^+]_{ext}$. Hereafter, each of these four sets of data will be referred to by number.

Admittedly, II could be explained on bases other than incompatibility of the Nernst hypothesis. For example, potassium ion at high concentrations may adhere to cell boundaries and be resistant to removal irrigation with low $[K^+]_{ext}$. However, I, III and IV could not be predicted by the Nernst equation. These data make its applicability to the cell membrane doubtful, since resistance and potential do not actually change simultaneously and promptly to changes in $[K^+]_{ext}$, as they do for inert membranes. What is more,

alteration of the response of cell membranes to $[K^+]_{ext}$ without apparent change in pore size is behavior peculiar to these cells. Pore size, estimatable from resistance, was not altered by this enzyme inhibitor, yet the membrane acted as though no K^+ were present even at concentrations up to 7 mM.

The data of arguments II and IV support an alternate explanation for the mechanism by which external K ion regulates membrane properties. This explanation was proposed as a result of experiments on muscle cells (Apter & Koketsu²). It appears that external potassium may become incorporated in the membrane as the result of some metabolic process. If so, it might be expected that potassium would be taken up faster in high $[K^+]_{ext}$ than it would be released in low $[K^+]_{ext}$. In addition, this new explanation is consistent with the finding that an enzyme inhibitor might prevent incorporation of K^+ in the membrane, thereby making the membrane insensitive to high K^+ .

This behavior resembles the action of cocaine and of tetraethylammonium ion, both of which make the muscle cell membrane less sensitive to $[K^+]_{ext}$ in a way inconsistent with the Nernst equation.

These data cannot yet contribute to knowledge of the manner by which a cell secretes a fluid. For this knowledge, it is necessary to make similar studies on the membrane of the other five sides of the cuboid epithelial cells, as well, probably, as of the pigmented epithelium. Only a first step toward this goal has been made in the present studies.

SUMMARY

1. With methods and equipment adequate for studying the properties of single muscle and nerve cells, individual cells in the single layer of non-pigmented epithelium of the eyes of bullfrogs, turtles and toads were impaled.

2. Membrane potential and resistance were measured and recorded simultaneously while

potassium ion concentrations in an external irrigating, Ringer's, solution were changed abruptly.

3. Potential had a negative relationship with potassium ion concentrations similar to muscle; resistance had a negative sigmoid relationship.

4. The time course of the response to an abrupt change in $[K^+]_{ext}$ was faster when concentrations were increased than when decreased; resistance changed more promptly than did potential.

5. Diamox converted both resistance and potential relationships to similar negative sigmoid curves. For an appreciable range of $[K^+]_{ext}$, the cell membrane maintained a potential and resistance level characteristic of solutions containing no K^+ .

6. These results indicate that the living cell membrane responds differently from an inert membrane to changes in potassium ion concentrations.

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DIABETIC RETINOPATHY

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Great progress in understanding diabetic retinopathy has been made within the last 20 years through studies like those of

Ashton,¹⁻⁴ Ballantyne,⁵ and Loewenstein,⁶ Day,⁷ Friedenwald,^{8,9} Hartford,¹⁰ O'Brien and Allen,¹¹ and Thiel.¹² In these studies examination of the whole unstained retina, blood vessel injection studies, many different histological staining techniques, and animal experiments have been used. The results are

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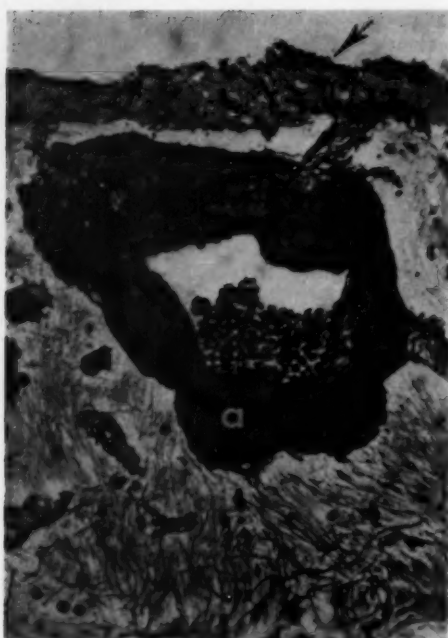


Fig. 1 (Wolter). Main branch of retinal artery with hyalinization of its wall (a) in diabetic retinopathy. The arrow indicates the inner limiting membrane at the disc margin. Flat frozen section, Hortege stain, photomicrograph.

very encouraging and it seems that the key to an insight into the cause of this diabetic complication could be found if we continue to collect details in applying all known special examination techniques to the retina in diabetes.

As yet, very little has been done to demonstrate the pathology of the main elements of the retina in diabetic retinopathy: the neurons, the nerves fibers, and the glia. A first try to fill this gap in our knowledge is made in the present paper. It is not intended here to give a complete review and discussion of the extensive literature on diabetic retinopathy. This paper as such is not a complete study of diabetic retinopathy. It should be considered part of and an addition to all that has been written about this complication by others.

Eight eyes of patients who died of dia-

betes at the Wayne County General Hospital at Eloise, Michigan, were used for this study. Most of the eyes were studied ophthalmoscopically before the death of the patient. There is no space in this paper for the details of the history and clinical findings of the cases used. It can be said, however, that all the eyes exhibited typical diabetic retinopathy and had no other eye diseases that we could see—except for diabetic cataracts in some of the cases.

Special stains must be used to demonstrate retinal neurons, nerves, and glia. The silver carbonate methods of del Rio Hortege¹³ represent the most reliable known techniques for the demonstration of these retinal structures. In the present study only the different variants of these techniques were used; sometimes in combination with a fat-O-red stain for the demonstration of lipids. All figures of this paper are unretouched photomicrographs.

FINDINGS

The retinal changes of advanced diabetic retinopathy as studied with the Hortege methods shows eight main lesions: the vascular damage, the proliferation of vascular (antidromic) nerves, the degeneration of neurons, the phagocytic activity of retinal microglia, the deposition of fat and hyaline, the retinal gliosis, the formation of drusen of the pigment epithelium, and punctate and diffuse retinal and preretinal hemorrhages. All these changes were seen in all the eyes with advanced diabetic retinopathy examined for this study. Thus, it seems practical to discuss the pathology of diabetic retinopathy in the order of these main changes listed above. It is important to emphasize that they are interdependent and are most likely all of the same primary cause which is still not understood.

VASCULAR PATHOLOGY

Sclerosis and hyalinization is an outstanding change of the wall of blood vessels in diabetic retinopathy. However, this is not

found in all retinal blood vessels to the same degree. Hyalinization is usually very advanced in the wall of all main branches of the central retinal artery and vein as they leave the optic disk (fig. 1). Of the smaller branches the arteries usually do not show much damage. Figure 2 shows a main arterial branch from a case of advanced diabetic retinopathy and demonstrates that the vessel wall is well preserved and virtually without fibrosis. The perfectly normal appearing longitudinal smooth muscle fibers of another retinal artery from a case of diabetic retinopathy are seen at high power in Figure 3. Much more sclerosis and hyalinization is found in the retinal veins and especially in the typical new-formed venous networks. (figs. 4 and 5).

Advanced changes in the veins and extensive new-formation of abnormal blood vessels (neovascularization) on the venous



Fig. 2 (Wolter). Main arterial branch of retinal artery in diabetic retinopathy. There is little pathology seen in this artery.—Flat frozen section, Hortege method, photomicrograph.



Fig. 3 (Wolter). Smooth muscle fibers of wall of main artery in diabetic retinopathy. These cells appear normal.—Frozen section, Hortege method, photomicrograph.

side of the retinal vascular system are well known typical features of advanced diabetic retinopathy. The networks of new-formed vessels include many of the so-called capillary microaneurysms (fig. 6). It is obvious from our slides that the neovascularization and the formation of microaneurysms goes on continuously in advanced diabetic retinopathy. All stages of the development of these structures are seen. It is important to emphasize that degenerative changes appear to occur in all these new-formed vascular structures soon after they are formed. Extensive deposition of fat, sclerosis, and hyalinization are seen in their walls and most of them are found to be totally obliterated. In the late stages of the degeneration of these new-formed vascular structures all that may remain of them are large hyaline bodies which represent totally hyalinized microaneurysms or remnants of other parts

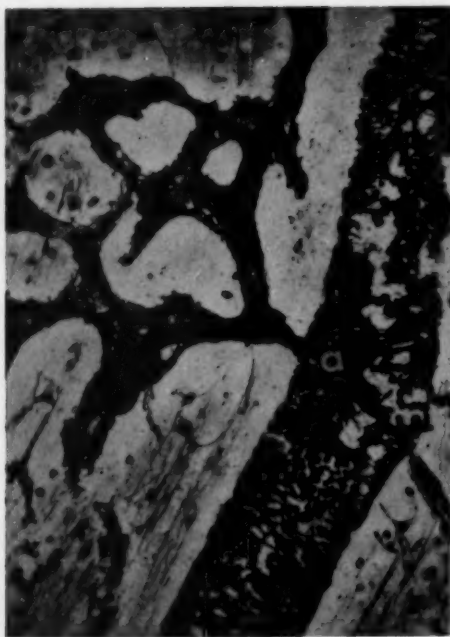


Fig. 4 (Wolter). Hyalinized venous network next to a larger vein (a) in diabetic retinopathy.—Flat frozen section of retina, Hortege method, photomicrograph.

of degenerated vessels of the venous side of the retinal blood vessel system (Wolter¹⁴). It is well known that the neovascularization in advanced diabetic retinopathy often extends through the inner limiting membrane of the retina into the vitreous space. This is usually seen in cases in which preretinal or vitreous hemorrhage has occurred.

A multitude of retinal microaneurysms in all phases of development is seen in all cases of diabetic retinopathy within the more or less pathological capillary network. In their early stages the microaneurysms have a large lumen filled with blood and a thin wall which is composed of endothelial cells (fig. 7). Soon fat deposition and fibrosis occur and result in thickening of their wall (fig. 8). Finally the whole wall becomes hyalinized and in the end stage the microaneurysms have only a small or no lumen (fig. 9).

It is surprising to see large areas of the capillary network in advanced diabetic reti-

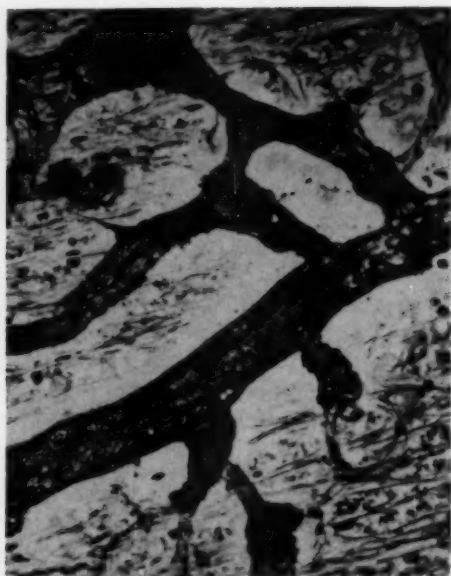


Fig. 5 (Wolter). Portion of hyalinized venous network in flat section of advanced diabetic retinopathy.—Frozen section, Hortege method, photomicrograph.

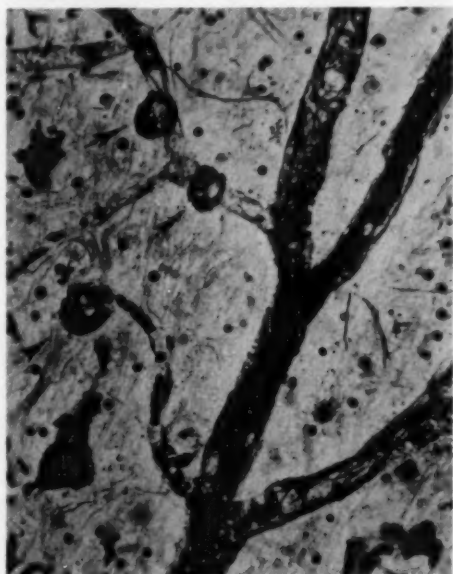


Fig. 6 (Wolter). View of flat section of retina with diabetic retinopathy exhibiting new-formed venous blood vessels containing microaneurysms (arrows).—Frozen section, Hortege method, photomicrograph.

nopathy with only little fat deposition in and around their walls which can be stained a bright red with a fat stain. In Figure 10 the fats were dissolved in the histological process and unstained empty spaces remain along the capillaries and are seen in the picture. It is mainly the venous side of the capillary system and the venules that exhibit the damage. In some areas of the retinas of our cases, however, there is total obliteration of all capillaries. This often involves small arterioles. Neovascularization may be found in these areas (Ashton¹).

Much thought has been spent to explain the development of the capillary aneurysms in diabetic retinopathy (Ashton¹). Recently Ashton (personal communication) has considered the possibility that retinal microaneurysms might be caused by the traction of peculiar bridge-like connective tissue strands in the process of general swelling

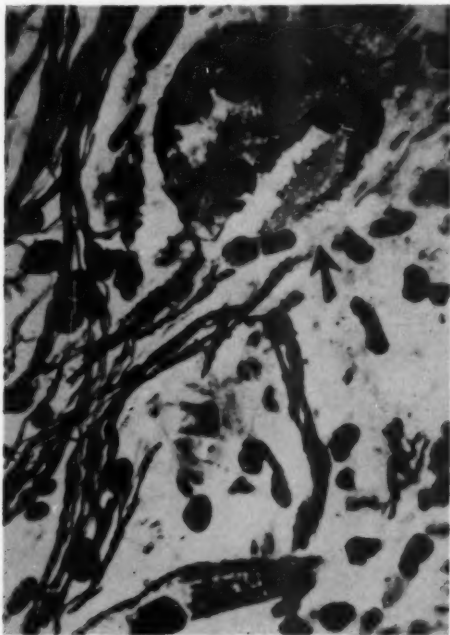


Fig. 7 (Wolter). Early stage of capillary microaneurysm (arrow) which is composed of flat endothelial cells just like those of the normal capillary wall.—Frozen section, Hortege stain, photomicrograph.

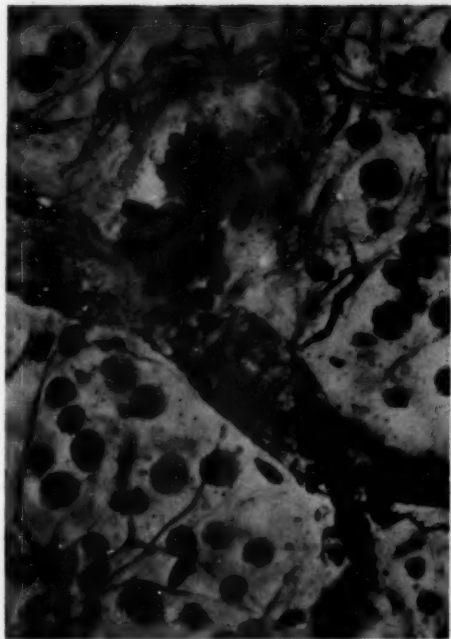


Fig. 8 (Wolter). A somewhat later stage of a diabetic capillary microaneurysms of the retina (arrow). Its wall is already thickened, but the lumen is still seen to be filled with erythrocytes.—Frozen section, Hortege stain, photomicrograph.

of the retina which is known to occur in diabetic retinopathy. Such bridge-like structures are known to interconnect small blood vessels in the brain Cajal,¹⁵ Wolter,¹⁶ optic

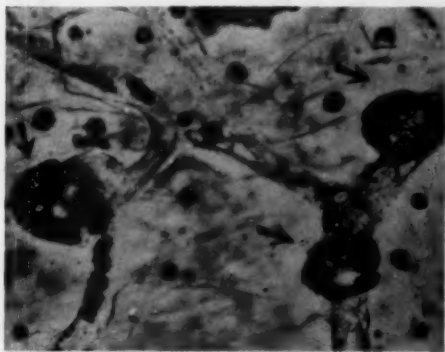


Fig. 9 (Wolter). Late stages of diabetic retinal microaneurysms (arrows). These have a thick hyaline wall and only a small or no lumen.—Frozen section, Hortege stain, photomicrograph.

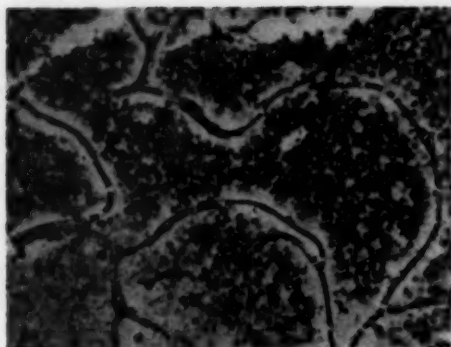


Fig. 10 (Wolter). Capillary network as seen in a case of advanced diabetic retinopathy in a flat section of the retina. The unstained empty spaces along the capillaries represent extensive fat deposition.—Frozen section, Hortege method, photomicrograph.

nerve (Wolter¹⁰) and in the retina (Wolter,¹⁰ Cogan¹⁷). Figure 11 shows a bridge-like connective tissue fiber in human diabetic retina between a larger vein and a small capillary. The nature of these intervascular connections is not yet understood. They are a normal finding in human brain, optic nerve and retina. It is conceivable that these structures are a kind of anchoring mechanism for blood vessels and might cause formations like retinal microaneurysms by direct pull on the vessel wall in swelling of the retinal tissues. However, so far our studies of

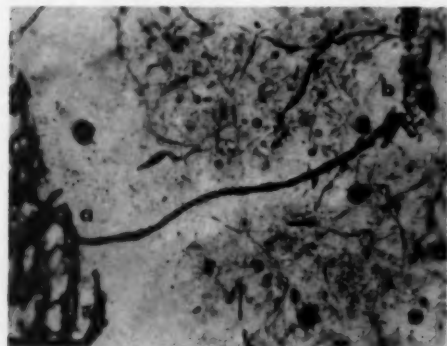


Fig. 11 (Wolter). Fibrous intervascular strand connecting a retinal vein (a) and a retinal capillary (b) as seen in a flat section of a human retina in diabetes.—Frozen section, Hortege stain, photomicrograph.

retinas with diabetic retinopathy exhibiting numerous microaneurysms have not revealed a single instance of a microaneurysm with one of these fibrous bands attached to it.* Figure 12 shows one of these fibrous interconnections next to but in no direct connection to two microaneurysms.

THE PROLIFERATION OF VASCULAR (ANTIDROMIC) NERVES

All neurons of the retina and the neurites of the nerve fiber layer and inner plexiform layer show extensive atrophy in advanced diabetic retinopathy. The centrifugal (antidromic) nerve fibers of the retina, however, make an exception. These nerves not only show no atrophy but it even appears that they proliferate in advanced diabetic retinopathy. This is a very surprising and probably also an important fact.

The centrifugal nerve fibers of the human retina come with the optic nerve from the brain.¹⁸⁻²¹ It is known that some of them at least supply the retinal blood vessels.²⁰ Figure 13 shows a centrifugal nerve fiber of this type in one of the diabetic retinas. The optic disc would be left in the pic-

* Further studies of diabetic retinas with the trypsin digestion technique of Kuwabara and Cogan revealed the attachment of fibrous bands to the microaneurysms in many instances (comp. Wolter, A.M.A. Arch. of Ophth. in print).

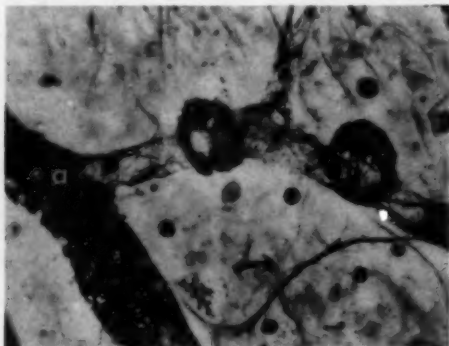


Fig. 12 (Wolter). Two hyalinized microaneurysms in the branches of a small vein (a). A fibrous intervascular strand (arrow) is seen next to the microaneurysms, but has no direct connections to them.—Flat frozen section, Hortege stain, photomicrograph.

ture and the fiber is seen to branch dichotomously twice in a direction away from the disc. The centripetal neurites of the nerve fiber layer which originate from the neurons in the ganglion cell layer never branch. Figure 14 shows two centrifugal nerves ending in the wall of a retinal blood vessel in a case of diabetic retinopathy (Wolter^{16,20}). The hyperplasia of these centrifugal vascular nerves in diabetic retinopathy is well demonstrated in Figure 15 many nerve fibers, one of them dichotomously branching, are seen around a slightly stained retinal blood vessel. Figures 16 and 17 show bizarre formations of coiled-up proliferated nerve fibers of this kind next to venous branches in advanced diabetic retinopathy. Such bizarre nerve fiber proliferations are well known as products of pathological nerve fiber regeneration from general and experimental neuropathology.

Proliferation of centrifugal nerves is not only found around retinal blood vessels but also around the microaneurysms. Figure 18 shows a retinal capillary in the earliest stage of microaneurysm formation with local dilation of the vessel and some increase of adventitial fibers. A nerve fiber is seen to run to and end within this dilated vascular area. Figure 19 shows a late hyalinized stage

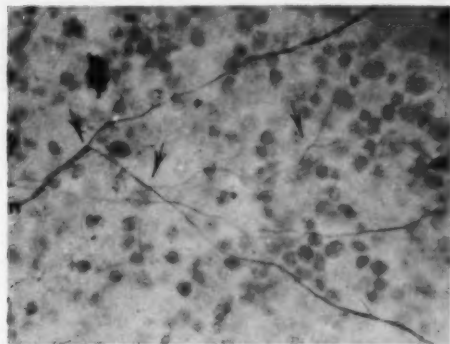


Fig. 13 (Wolter). Branching centrifugal nerve fiber of the human retina in a case of diabetic retinopathy. Arrows indicate the points of nerve fiber branching. The optic nerve would be on the left side of the photographed area.—Flat frozen section, Hortega method, photomicrograph.

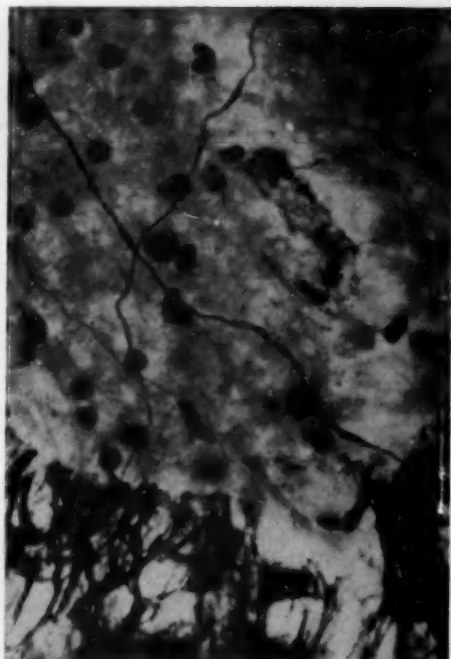


Fig. 14 (Wolter). Centrifugale vascular nerves entering the wall of a retinal vein (below in picture) in diabetic retinopathy.—Frozen flat section, Hortega method, photomicrograph.

of a retinal microaneurysm with centrifugal nerves coiled around it. One of the nerves shows a dichotomous branching. Figures 15 to 19 show evidence of a peculiar prolifera-

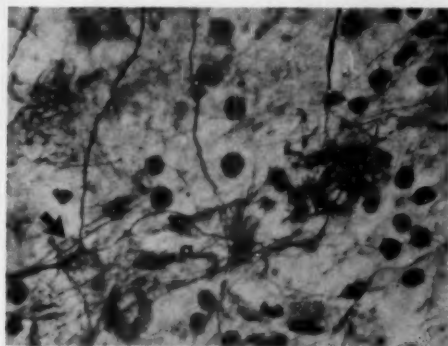


Fig. 15 (Wolter). Numerous nerve fibers around small retinal vessel (a). One of the nerve fibers is seen to branch dichotomously (arrow).—Frozen section, Hortega stain, photomicrograph.

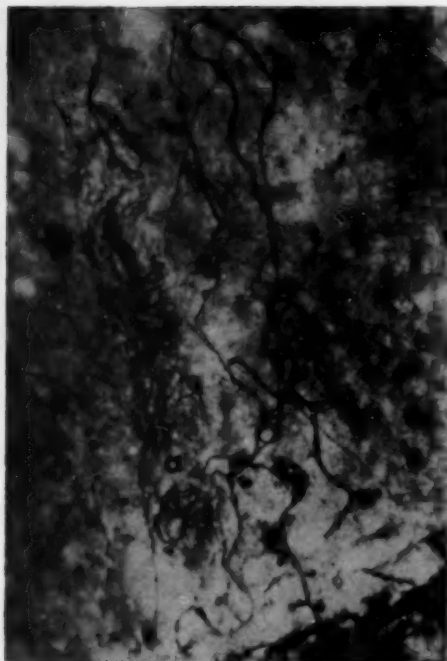


Fig. 16 (Wolter). Coiled-up hyperplastic nerve fiber seen next to and in connection with a small venous branch (a) of a larger retinal vein (b) in diabetic retinopathy. The coiled-up nerve is branching at many points of its course.—Frozen section, Hortega method, photomicrograph.

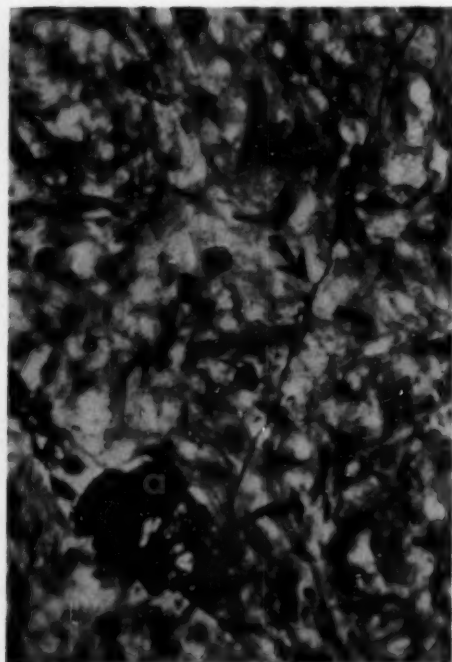
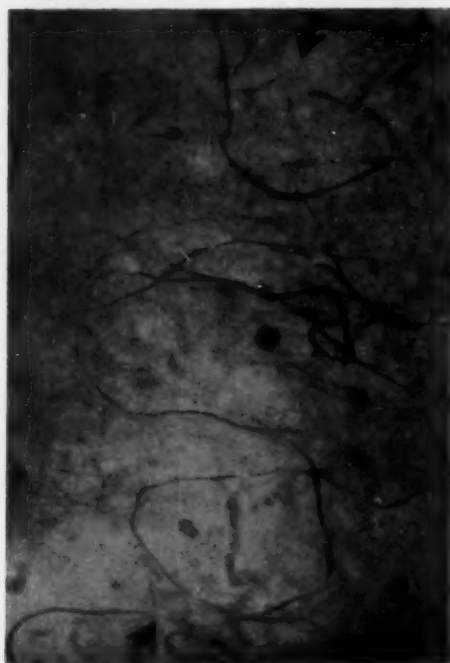


Fig. 18 (Wolter). A nerve fiber (arrow) supplying a small capillary microaneurysm in diabetic retinopathy (a).—Frozen flat section, Hortega stain, photomicrograph.

Fig. 17 (Wolter). Hyperplastic and pathologic nerve fibers coiling around a retinal vein in diabetic retinopathy. The vein itself is not seen in this part of the slide. Its direction is indicated by the arrows.—Frozen section, Hortega stain, photomicrograph.

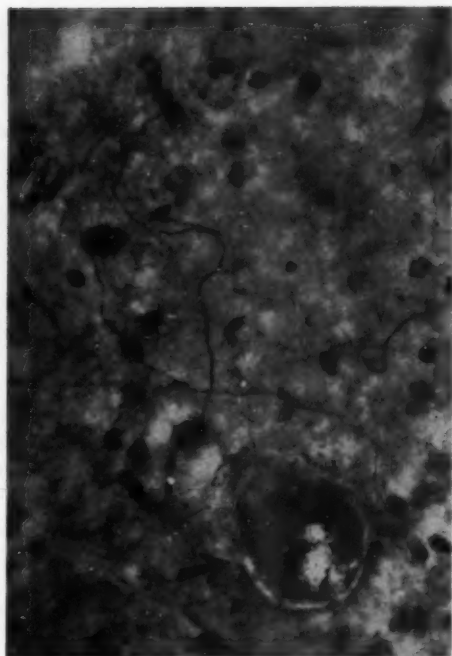


Fig. 19 (Wolter). Late stage of a hyalinized microaneurysm (arrow) in diabetic retinopathy with pathological nerve fibers coiling around it.—Frozen section, Hortege method, photomicrograph.

tion of the centrifugal retinal nerves which supply the blood vessels, including the new-formed vascular networks and microaneurysms. The apparent increase of these

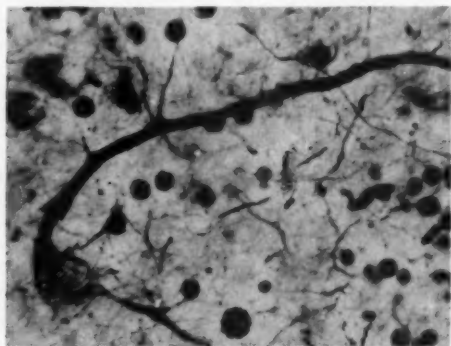


Fig. 20 (Wolter). Degenerating ganglion cell in diabetic retinopathy. The cell body and the neurite are fragmented. The dendrites are swollen.—Frozen section, Hortege stain, photomicrograph.

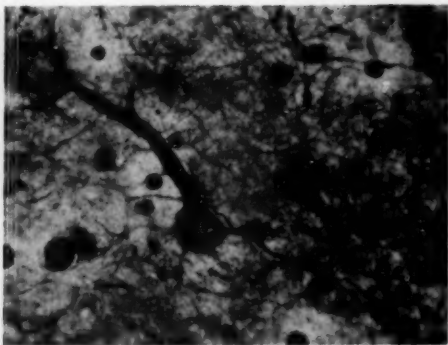


Fig. 21 (Wolter). Degenerating retinal ganglion cell in diabetic retinopathy. The cell and its dendrites are swollen. Fragments of other degenerating ganglion cells are seen around it.—Frozen section, Hortege stain, photomicrograph.

nerves and their abnormal and bizarre arrangement around blood vessels and microaneurysms are seen in all our retinas with advanced diabetic retinopathy.

DEGENERATION OF THE NEURONS

Diabetic retinopathy always goes along with progressive degeneration of the retinal neurons, including their neurites and dendrites. This can be considered as the direct reason for the loss of vision that is observed in diabetic retinopathy. A multitude of degenerating neurons, each of them exhibiting a somewhat different stage and pattern of slowly progressive degeneration, are seen in

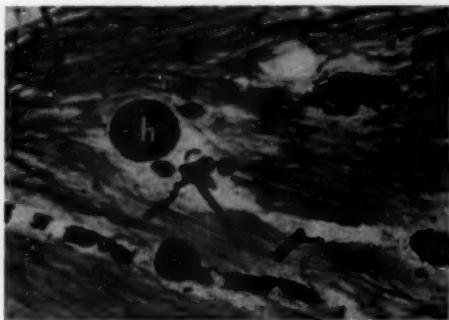


Fig. 22 (Wolter). Hyaline body (h) of nerve fiber origin in the nerve fiber layer close to the disk in diabetic retinopathy.—Frozen section, Hortege method, photomicrograph.

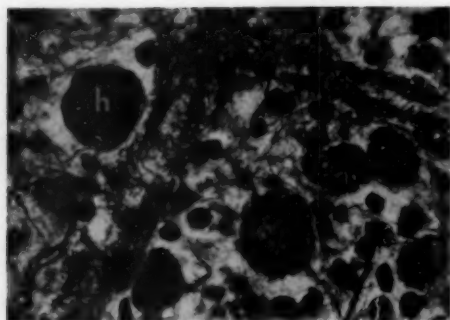


Fig. 23 (Wolter). Hyaline body (h) of ganglion cell origin in the ganglion cell layer in diabetic retinopathy.—Frozen section, Hortege stain, photomicrograph.

all advanced cases of diabetic retinopathy. The limits of this paper and the number of illustrations allow only for some examples of this degeneration. Typical stages in the destruction of large neurons are seen in Figures 20 and 21. The cells and some of their processes are swollen. Others are fragmented and pieces of neurites and dendrites as well as of the cell bodies are seen in the surrounding tissue. Dissolution of cellular nuclei and cell bodies is seen (fig. 20). Finally in this process the cells disappear and their debris are phagocytized and removed by retinal microglia. Hyalinization is also a common type of ganglion cell degeneration in diabetic retinopathy (Wolter²²). Round hyaline bodies are all that finally remains of the cell bodies of the

neurons in this type of degeneration (fig. 22). It is typical of advanced diabetic retinopathy to see a tremendous decrease of the number of neurons especially in the inner layers of the retina. Consequently there is also a decrease of nerves in the nerve fiber layer of these retinas. Hyaline bodies of nerve fiber origin (Wolter²³) are usually seen all through the nerve fiber layer and the optic disc (fig. 23). Cotton wool spots composed of interrupted nerves of the nerve fiber layer with terminal swellings of Cajal (Wolter²⁴) also are a common finding in diabetic retinopathy. Nerve fiber stumps with their cell-like (cytoid) terminal bodies are also found diffusely as single occurrences all through the nerve fiber layer (fig. 24a). It is important to emphasize that all the nerve fibers of retinal origin have their terminal swellings pointing towards the optic disc. However, rarely there are also nerve fiber stumps with much smaller terminal swellings which point away from the optic disc. (fig. 24b). These are considered to be the stumps of degenerated centrifugal nerves of the retina.

PHAGOCYTTIC ACTIVITY OF RETINAL MICROGLIA AND THE DEPOSITION OF FAT AND HYALINE

The debris of the degenerating retinal neurons in diabetic retinopathy are phagocytized by retinal microglia. After phagocytosis these mesodermal cells represent

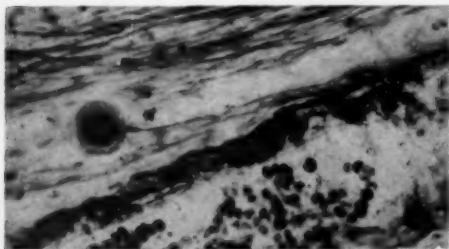


Fig. 24a (Wolter). Terminal swelling of Cajal of an interrupted fiber in the nerve fiber layer (arrow) pointing towards the optic disc (centripetal, afferent nerve). A blood vessel is seen next to it.—Hortege stain, photomicrograph.

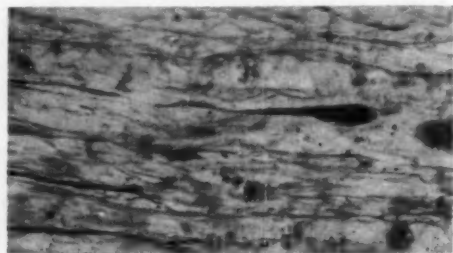


Fig. 24b (Wolter). Terminal swelling of a thin nerve fiber pointing in the opposite direction (arrow) —away from the optic disc (centrifugal, efferent fiber)—in diabetic retinopathy.—Frozen section, Hortege stain, photomicrograph.

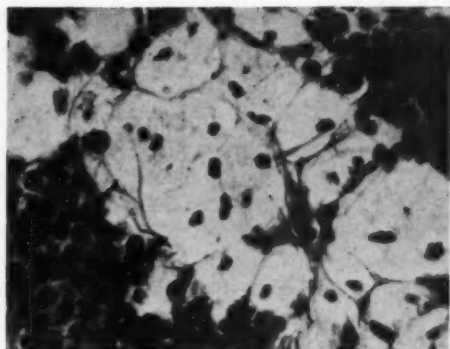


Fig. 25 (Wolter). Accumulation of fat-filled microglia (gitter cells) in the ganglion cell layer of the fovea in diabetic retinopathy.—Flat frozen section, Hortege stain, photomicrograph.

large fat-filled cells with a small round nucleus (gitter cells). In other diseases of the retina in which there is destruction of

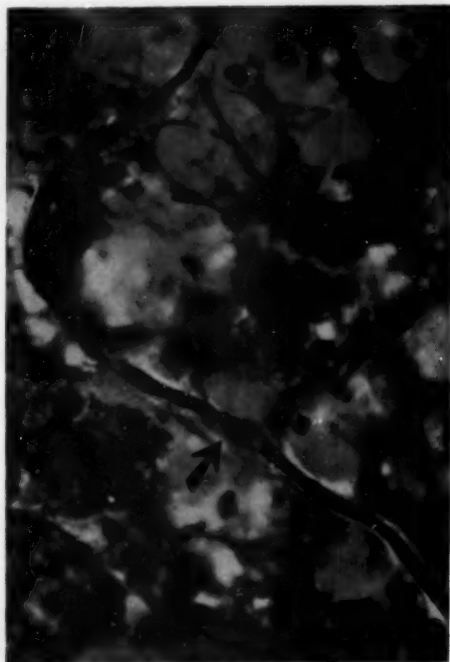


Fig. 27 (Wolter). Capillary (arrow) of the retina in diabetic retinopathy surrounded by many fat-filled microglia (gitter cells).—Flat frozen section, Hortege stain, photomicrograph.

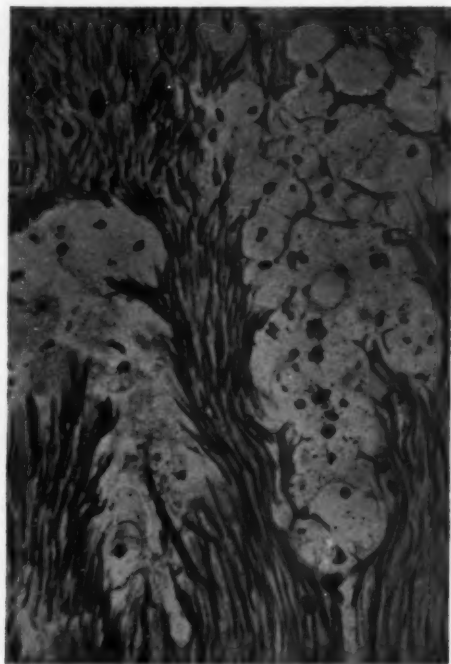


Fig. 26 (Wolter). Accumulation of fat-filled microglia (gitter cells) in Henle's fiber layer of a case of advanced diabetic retinopathy.—Frozen flat section, Hortege stain, photomicrograph.

retinal neurons these elements also phagocytize the neuronal debris then move to the blood vessels and are removed from the retina. This process of delivery of the neuronal debris (lipids and proteins), however, is somehow disturbed in diabetic retinopathy. The debris-filled microglia obviously cannot deliver the phagocytized substances to the blood stream. Thus, these cells remain in the retina. They pile up in the macular area (figs. 25 and 26) and also, of course, around the retinal capillaries (fig. 27) and microaneurysms (fig. 28). Henle's fiber layer is an area that shows accumulation of the fat-filled cells relatively early in many cases of diabetic retinopathy (fig. 26). The accumulation of these cells goes along with extensive degeneration of Henle's fibers. In the late stages of diabetic retinopathy fat-filled microglia are seen all

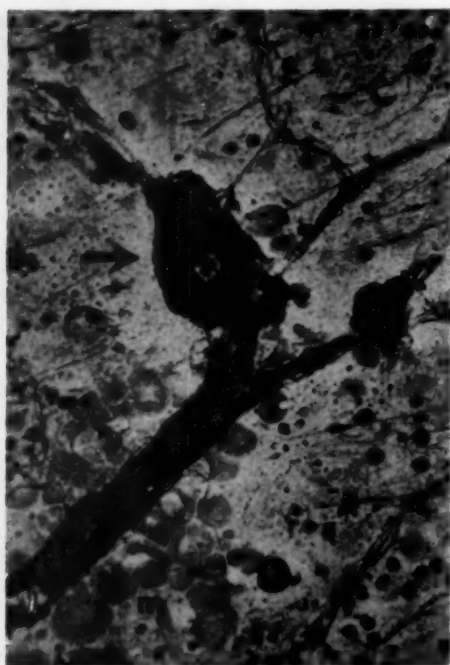


Fig. 28 (Wolter). Fat-filled microglia (m) piled up around a hyalinized vein and microaneurysms in diabetic retinopathy.—Frozen section, Hortege stain, photomicrograph.

through the retina. The fat-filled and deposited macrophages seem to have only a certain life time, just like erythrocytes and other free cells of the organism, and thus they may be seen in all stages of degeneration. Large pools of free lipids (contain also proteins) mainly in and around the fovea are the result of this degeneration of the accumulated lipid-filled cells. This remaining extracellular substance may then become hyalinized. This hyalinization usually starts out from round islands of hyaline which appear in the middle of the lipid containing free substance. Figure 29 shows a flat section of the central retina in advanced diabetic retinopathy at low power. Accumulated fat-filled microglia in all phases of degeneration, free lipids ("exudates"), areas of hyalinization, and a microaneurysm surrounded by fat are seen. It is interesting that re-phagocytosis of the deposited free fats and pro-

teins in the retina of advanced retinopathy could not be observed in our cases.

GLIOSIS

As usual in retinal pathology proliferating astroglia forms a scar to fill the space of the degenerating neurons in diabetic retinopathy. This scar of hyperplastic astrocytes surrounds the new-formed blood vessels and microaneurysms (fig. 30). Virtually no ganglion cells are left in many areas of this glial scar (fig. 31). The radial fibers of Mueller are not seen to take an active part in this glial scar formation.

FORMATION OF DRUSEN OF THE PIGMENT EPITHELIUM

Drusen of the pigment epithelium are a typical finding in diabetic retinopathy. They are usually of a rather dense arrangement

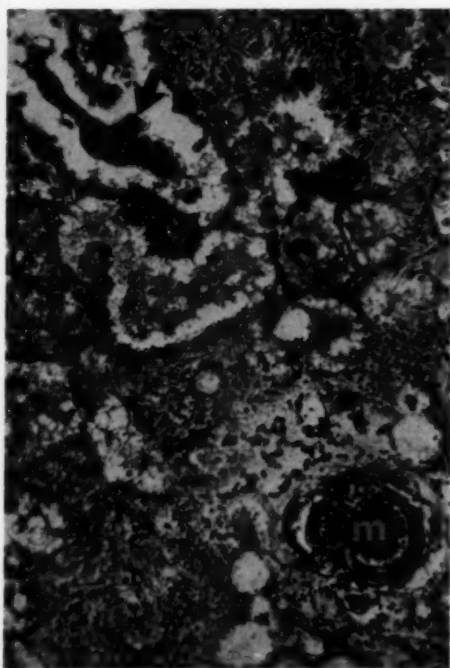


Fig. 29 (Wolter). Area of a flat section through the central retina in diabetic retinopathy. There are large accumulations of free lipid and fat-filled microglia (i), a microaneurysm (M), and areas of hyalinization (arrow).—Frozen section, Hortege stain, photomicrograph.

(fig. 32). In their early stages they are positive to a fat stain; in their late stages they are composed of hyaline and do not take a fat stain. With high power magnification the Drusen show a lamellar structure with a dense darker staining center (fig. 33).

PUNCTATE AND DIFFUSE RETINAL HEMORRHAGES

"Punctate" retinal hemorrhages are very typical of diabetic retinopathy of all phases. They are found in the inner nuclear or inner plexiform layers (figs. 34 and 35). They are of irregular shape but usually rather well circumscribed. Glial scarring is seen to surround them in their later stages. They are distinctly different from retinal microaneurysms.

Diffuse or radiating intraretinal hemorrhages as well as preretinal hemorrhages also are found commonly in cases of more advanced diabetic retinopathy.



Fig. 30 (Wolter). Hyperplastic astroglia of retina in diabetic retinopathy seen to surround a microaneurysm (dark area in lower part of picture).—Frozen section, Hortege stain, photomicrograph.

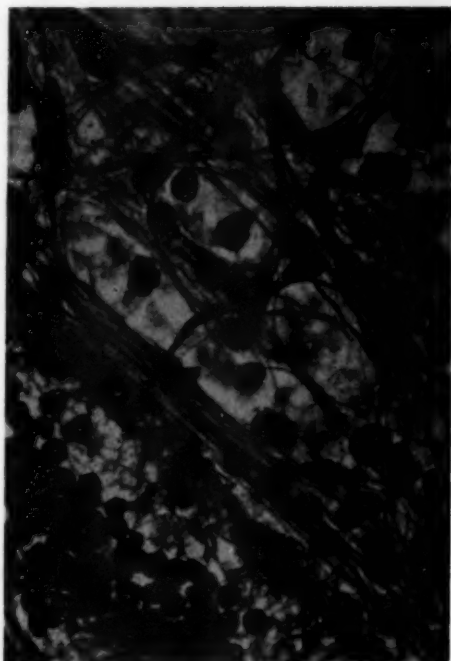


Fig. 31 (Wolter). High power view of hyperplastic astroglia next to retinal artery.—Frozen section, Hortege stain, photomicrograph.

DISCUSSION

This study confirms the well known fact that the vascular changes of advanced diabetic retinopathy mainly involve the venous side of the capillary system and the venules. The capillaries and the arteries show in most of the cases relatively little damage except for varying fibrosis and hyalinization. Localized secondary obliteration of retinal vascular areas including the arterioles may be seen in late stages of diabetic retinopathy. Friedenwald^{8,9} and Day⁷ have shown that the wall (basement membrane) of the diabetic retinal vessels contains large amounts of mucopolysaccharides. It is typical that virtually all the new-formed vessels on the venous side of the capillary system are abnormal. Soon after they develop, they seem to become irregular in caliber and show extensive fibrosis and hyalinization of their walls. Thus, in advanced diabetic retinopathy there still seems to be a lack of functioning

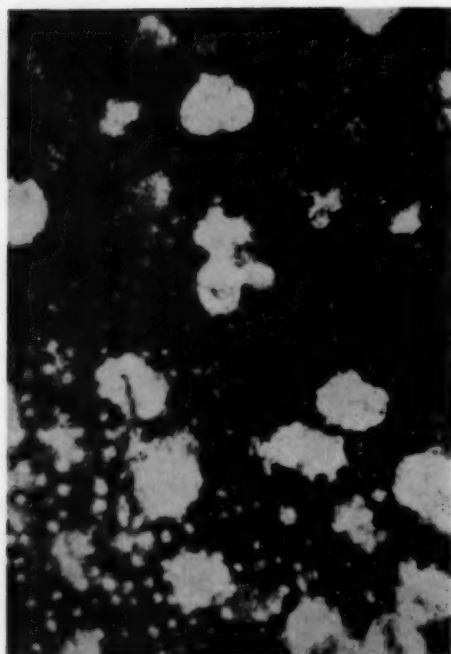


Fig. 32 (Wolter). Flat section through the pigment epithelium of a case of diabetic retinopathy shows many drusen of Bruch's membrane (unstained areas).—Flat frozen section, Hortega stain, photomicrograph.

blood vessels of the small venous kind despite constant new-formation of these vessels.

Microaneurysms also occur mainly on the venous side of the capillary network. In their early phases microaneurysms represent saccular formations of vascular endothelium with some hyperplastic adventitial fibers. Soon after their development, however, fat deposition and thickening in their wall occurs. This thickened wall then becomes hyalinized. Finally the lumen becomes smaller and smaller; and in their final stage microaneurysms represent solid hyaline bodies without a lumen. Usually the adjacent capillaries within which the microaneurysm developed also become hyalinized and obliterated.

Fat stain reveals that much lipid material is deposited in the wall of all retinal

capillaries in advanced diabetic retinopathy. Even more fat is usually seen in the walls of and around microaneurysms.

Several theories have been suggested to explain the development of retinal microaneurysms. However, it seems to me that there is no theory yet into which all observed clinical and pathological details fit. In looking for the direct cause of retinal microaneurysms Ashton²⁵ has recently considered a new and very interesting concept. It is known that retinal blood vessels just like those of the brain and optic nerve have peculiar, course, fibrous interconnections. It occurred to Ashton that these bridge-like interconnections might cause the typical kinking seen in the early stages of the formation of microaneurysms by direct pull. This explanation appeared so simple and convincing that all our flat sections of human

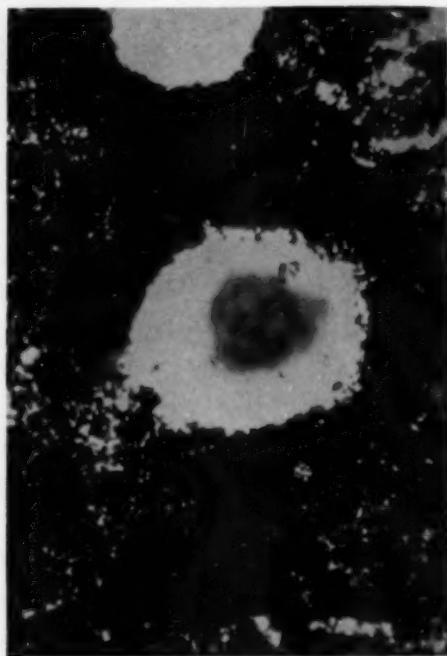


Fig. 33 (Wolter). High power view of a druse in the pigment epithelium with its denser hyaline nucleus.—Frozen section, Hortega stain, photomicrograph.

retinas with diabetic retinopathy were studied again to see whether evidence could be found for this theory. Many microaneurysms and many of the bridge-like intervascular strands were seen. However, in no instance was a microaneurysm or the kinking of a capillary found to be related to the insertion of the intervascular strands.*

In staining for and studying the bridge-like intervascular strands described above I discovered another histological finding that may well be of importance. This is an extensive proliferation of the centrifugal nerves of the retina which are known to supply the human retinal blood vessels.¹⁸⁻²¹ In all our cases of advanced diabetic retinopathy there was a distinct increase in the

* A relation of intervascular strands to diabetic microaneurysms is demonstrated in a later paper (Wolter, A.M.A. Arch. of Ophth. in print).

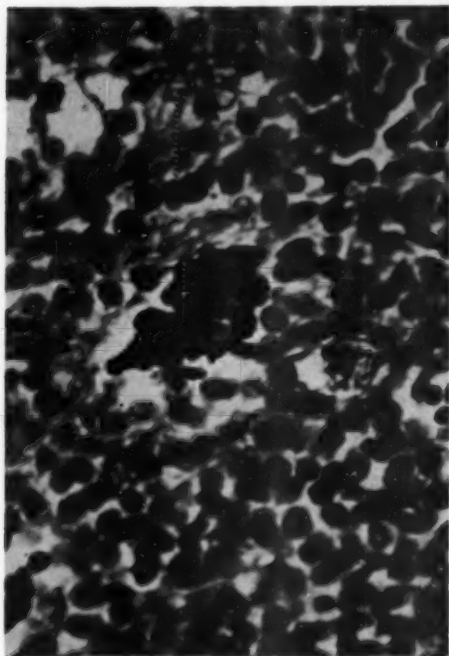


Fig. 34 (Wolter). Small "punctate" hemorrhage in inner nuclear layer of a case of diabetic retinopathy.—Flat frozen section, Hortege method, photomicrograph.



Fig. 35 (Wolter). Larger "punctate" hemorrhage in the inner plexiform layer of a case of diabetic retinopathy.—Frozen section, Hortege method, photomicrograph.

relative number of the centrifugal fibers as compared with the centripetal fibers. I am not able to say whether there actually was an increase of the centrifugal nerves. One fact, however, is certain: in all retinas there was a very advanced atrophy of the ganglion cells and their neurites (centripetal nerves), while the centrifugal nerves showed only slight atrophy even in the most advanced cases. Furthermore, there always was an increase and pathologic proliferation of the retinal branches of the centrifugal nerves which supply the blood vessels. The centrifugal nerves could be well observed with their dichotomic branchings pointing away from the optic disc. Their courses often crossed or ran at an angle to the general direction of the fibers in the nerve fiber layer. Around the retinal blood vessels, especially around the smaller veins, the new-formed networks, and the microaneurysms,

the branches were seen to form bizarre nerve formation. These looked much like the products of pathologic nerve regeneration and proliferation as known from general and experimental neuropathology.

It cannot yet be said how important this finding of a pathologic vascular innervation in advanced diabetic retinopathy is. It could be very important since it is possible that the nerve damage is primary and a cause of the vascular changes. However, there is no proof of this in my slides.

The first change to occur in the retina in diabetes, as far as I know, is the swelling and degeneration of retinal neurons. This involves mainly the neurons of the inner retina layers with all their processes. A multitude of different types of neuronal degeneration is seen and it would take a special paper to describe some of the more common types of slowly progressive neuronal destruction. In this paper it is important, however, to emphasize that most types of neuronal degeneration in diabetic retinopathy finally end in a break-down of the cells and their processes. Their debris remain and are, of course, proteins and mainly lipids.

The retinal nerve fibers take part in the degeneration process. Cotton wool spots²⁴ and diffuse single nerve stumps with terminal swellings (cytoid bodies) are always present. Hyaline bodies are also common and may develop from ganglion cell or nerve fibers.^{22,23}

It is well known that tremendous numbers of fat-stain-positive round cells, called ghost cells or spectral cells, are found in the retina in all advanced cases of diabetic retinopathy. It is also well known that these cells especially occur in the central retina within areas of "exudates" that contain much fat and hyaline. After studying flat sections of diabetic retinopathy my view of the role of the retinal macrophages in this process is somewhat different than the usual one. I will explain it shortly: the primary retinal pathology in diabetes mellitus seems

to be neuronal. The neurons finally break down and their debris are phagocytized by retinal microglia (macrophages). In the process of phagocytosis the microglia become large round cells known in neuropathology as gutter cells or granular compound corpuscles.^{26,27} They are the ghost cells of the ophthalmological literature. These gutter cells stain a bright red with any fat stain. They are, after all, filled with the lipoidal debris of the destroyed retinal neurons. For an unknown reason these fat-loaded cells, or at least many of them, cannot deliver the phagocytized debris to the blood stream as they do in many other pathologic processes with destruction of retinal neurons. They pile up in the retina, and this piling up usually first occurs in the foveal area. I have seen several cases of diabetic retinopathy where the so called deep hard exudates were composed of such fat-filled microglia only and where there was no primary exudate. Later in the process when more and more fat-filled gutter cells accumulate in the retina some of them start to degenerate. The substances which they contained remain as free fluid in the retina. It stains a bright red with a fat stain. For an unknown reason this free substance is not rephagocytized but it becomes slowly hyalinized. Thus, it seems that the development of the so-called deep hard exudates and the star figure in diabetic retinopathy is much like that of similar formations in hypertensive retinopathy.^{28,29} The difference in the pattern of the star figure in diabetic and hypertensive retinopathy might be caused by the fact that the capillary system in diabetic retinopathy has become abnormally rearranged in the process of neovascularization.

Not much has to be said about the gliosis of the retina in diabetic retinopathy. Glia always forms a dense retinal scar when a chronic process slowly destroys the neurons.²⁷ The glia also surrounds the new-formed blood vessels, the microaneurysms, and even the retinal hemorrhages with dense

fibrillar networks. There is no doubt that punctate hemorrhages do occur in the diabetic retina. It is not true that all red spots seen with the ophthalmoscope are microaneurysms. On the other hand it should be emphasized that only the recently formed microaneurysms which still have a lumen and are filled with erythrocytes can be seen as red spots with the ophthalmoscope. Old microaneurysms obliterate and cannot be expected to appear as red spots in the fundus.

Diabetic retinopathy in its late stages always goes along with extensive degeneration of the pigment epithelium. It exhibits irregular loss of pigment granules, deposition of fat, and development of hyaline Drusen in and beneath the pigment epithelium.²⁰ The choroid of all cases showed only extensive vascular sclerosis in most of them.

Finally I would like to emphasize that all the described pathology of diabetic retinopathy, with the exception of the abnormal proliferation of the vascular nerves, has to be considered non-specific. It is well known that microaneurysms may occur in other pathologic entities of the retina (Ashton,^{1,3} Becker and Post²¹). The same is true with neovascularization, all types of hemorrhages,

destruction of neurons, piling up of microglia, reposition of fat and hyaline, and gliosis. The pattern and type of all these changes together, can be called typical of diabetic retinopathy. The proliferation of the vascular nerves of the retina is the only single finding that has not yet been observed in any other condition of the retina.

SUMMARY

The pathology of the retina in diabetes mellitus was studied with flat sectioning and silver carbonate staining technique in eight eyes of patients who had died in advanced stages of the disease. The retinal changes discussed in this demonstration include: vascular pathology, proliferation of the vascular (antidromic) nerves, degeneration of the neurons, phagocytic activity of retinal microglia, deposition of fat and hyaline, retinal gliosis, formation of drusen of the pigment epithelium, and punctate and diffuse retinal and preretinal hemorrhages. All this, with the exception of the abnormal proliferation of the vascular nerves, has to be considered non-specific.

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DISCUSSION

A. EDWARD MAUMENE (Baltimore): Dr. Wolter has demonstrated to us once again that when a new technique is used to study the eye, interesting observations can be made. It was pointed out in Penfield's book on Cytology and Pathology of the Nervous System in 1932, that few studies had been made on the neurons, nerves and glial tissues of the retina by the silver technique, in pathologic conditions. Dr. Wolter's numerous publications on this and related subjects have proven its value.

The concept proposed in this presentation, that the retinal changes other than the proliferation of the vascular nerves of the retina are non-specific is an interesting one, for it would indicate two things. One that this proliferation of the vascular nerves is in some way responsible for or related to the cause of the diabetic retinopathy. This is the first time I have heard such a concept expressed and I would like to know if the author has any explanation of why this specific proliferation of vascular nerves should occur in diabetic patients. Next, I have thought that the pattern of vascular changes that occurs in the retina, was specific for diabetes. It is true that microaneurysms in the retinal vessels have been found in lesions other than diabetic retinopathy. However, in these other conditions they are usually much fewer in number and occur only when there has been extensive vascular damage from other causes. Also, in these conditions the microaneurysms usually seem to be a secondary lesion and play only a passive role in the progress of the disease. In diabetes, on the other

hand, the first clinically observable lesions appear to be the microaneurysms in an otherwise normal fundus. A progression of this vascular abnormality thus leads to the other changes in the fundus.

Although the cause of the vascular changes in the eye and kidney are not known at this time, it is suspected that they may be due to an alteration of the mucopolysaccharide or fat metabolism in the diabetic. An interesting report appeared in the 1960 January-February issue of *Diabetes* by Pope, in which he attempted to show that the pathogenesis of the microaneurysm in diabetic retinas was the accumulation of fat in the capillary wall with subsequent stretching, so that it herniates through the supporting reticulum network. A minute aneurysm then develops by this herniation between reticulum fibers.

Dr. Wolter's findings that microaneurysms did not occur where the intervascular bridges attach to the walls of the vessels eliminates an interesting mechanical means by which these aneurysms might have been formed.

I would like to know if the author has any explanation of how the hard exudates are absorbed when they disappear from clinical view, if he does not think that the lipid and hyalin deposits are rephagocytized after the microglia that originally devoured these substances are destroyed?

Finally, I would like to congratulate the author on his beautiful preparations of silver stains of the retina. These are certainly amongst the best that appear in the literature. He has mentioned many

times that he uses the technique of Sharenberg to prepare this material. I wonder if it is necessary to modify in any way the technique described in detail by Sharenberg in the 1954 volume of the *American Journal of Pathology* to obtain such nice preparations of the retina?

DR. WOLTER (closing): May I first thank Dr. Maumenee for his kind words.

In answering Dr. Maumenee's questions I would like to state that I believe that vascular pathology in general could occur as a result of pathology of the supplying nerves. However, our slides show no evidence that the nerve pathology is primary. The fact that the vascular nerves in the diabetic retina show pathology is not very surprising since neuritis and other nerve pathology is commonly found in diabetes.

I agree with Dr. Maumenee's observation that the pattern of the vascular changes in diabetic retinopathy is specific for diabetes. However, the components of this pattern appear to be nonspecific.

As to the question about the "hard exudates" I should have said that these usually do not become rephagocytosed in advanced retinopathy. In unusual cases they may disappear and it seems that this only occurs if the primary vascular pathology improves, which is rare in advanced diabetes.

I use the silver carbonate techniques of Horteaga as they were modified by Scharenberg. However, Horteaga and Scharenberg have often pointed out that the methods always have to be adjusted to a given tissue. Scharenberg stated in his 1952 paper that his methods are only suggestions from which one may start out to learn silver staining. The description of the methods have about the same relation to a successful stain as the brush and the paint have to a good painting. Silver staining cannot very well be done by a technician. The retina offers a special challenge since only frozen sections can be used for silver staining and since the retina has to be cut in flat sections for most of our studies.

THERMODYNAMICS OF THE INTRAOCULAR AND EXTRAOCULAR MUSCLES*

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Chicago, Illinois

Experiments recently completed indicate that resting muscle, smooth and striated, responds to temperature changes in a predictable and systematic way (Apter^{1,2,3}). The temperature-tension correspondence fit a negative sigmoid curve of the following form:

$$f = \frac{f_{\max} e^{C(1/T - 1/T_0)} + f_{\min}}{1 + e^{C(1/T - 1/T_0)}} \quad (1)$$

The term, f , refers to the force exerted by muscle exposed to absolute temperature, T ; f_{\max} and f_{\min} are the upper and lower asymptotes of the sigmoid curve, occurring at 6.0°C and 37°C respectively; T_0 is the absolute temperature at which the point of inflexion occurs, and C is the slope of the rectified data.

The data rectified to a log-reciprocal plot, giving C as follows:

$$\log \frac{f_2 - f_{\min}}{f_{\max} - f_2} - \log \frac{f_1 - f_{\min}}{f_{\max} - f_1} = C \left(\frac{1}{T} - \frac{1}{T_2} \right) \quad (2)$$

Converting to natural logarithms, and expressing (equation 2) as an integral equation,

$$\int_{T_1}^{T_2} \frac{d \ln \frac{f - f_{\min}}{f_{\max} - f}}{dT} = 2.3C \int_{T_1}^{T_2} \frac{1}{T^2} \quad (3)$$

This behavior of muscles could not be explained by properties of inert elastic elements in muscle. In addition, mere changes in elasticity coefficients of either relaxed or contracted contractile elements were inconsistent with some of the data.³ Instead, the force at f_{\max} was most likely a measure of contracted contractile elements (A_e) which existed in the relaxed form (A_r) at f_{\min} . At other points on the sigmoid temperature-tension curve

$$f - f_{\min} = kA_e \quad (4)$$

where k is the proportionality factor, and A_e is the concentration of the contractile

* This work was supported by United States Public Health Service grant No. B-1177 (C3), and by a grant from the State of Illinois Department of Public Welfare.

element in the "contracted form." Furthermore, let

- A_r = concentration of "relaxed form."
 f_o = contraction tension per mole of A_o
 f_r = contraction tension per mole of A_r
 $S = A_o + A_r$ = total of "contracted" and "relaxed" forms.

This last assumption follows from the reversibility of the temperature-tension relationship. Then

$$f_{\max} - f = kA_r \quad (5)$$

leading to

$$\frac{f - f_{\min}}{f_{\max} - f} = \frac{A_o}{A_r} \quad (6)$$

This ratio may then act like an equilibrium constant, K , in a postulated reaction



giving

$$\frac{A_o}{A_r} = K \quad (8)$$

$$A_o = \frac{KS}{1+K} \quad (9)$$

$$A_r = \frac{S}{1+K} \quad (10)$$

Substituting

$$K \text{ for } \frac{f - f_{\min}}{f_{\max} - f}$$

in equation 3 gives the van't Hoff equation if C is $\frac{\Delta H}{R}$. From the slope of a

plot of $\log K$ against the reciprocal of the absolute temperature, ΔH could be calculated. This plot for the intraocular and extraocular muscles of cats and dogs is depicted in Figure 1. ΔH is $-29,900$ cal/mole (\pm s.d. 2,700). The negative sign indicates that the transformation from relaxed to contracted state is exothermic. Indeed, heat production measurements on other muscles indicate that heat is produced during the phase of contraction.^{9,7,11} At T_o , S is -108 cal/mole/degree. This negative sign indicates that relaxed elements are at higher entropy than are contracted

elements; are more disorganized when relaxed. X-ray diffraction^{4,13,14} and electron microscope studies¹⁷ also suggest that contractile elements are more disorganized in the relaxed form. The present data are not consistent, however, with the concept of a long chain molecule which folds upon contraction.^{5,8,19}

The intraocular smooth muscles (not the spurious "dilator pupillae") responded to graded concentrations of methacholine (mecholy) with an exponential relationship as follows

$$f(M)_T = f(M)_{XT} [1 - e^{-(M-M_o)/(1.4(M_{50}-M_o)}] \quad (11)$$

$f(M)_T$ is the force at temperature T induced by mecholy concentration M ; $f(M)_{XT}$ is the force at the asymptote of this exponential dose-tension curve; M_o is the threshold dose of mecholy and M_{50} is the dose inducing 50 percent of $f(M)_{XT}$. These muscles gave a family of sigmoid temperature-tension curves with a common f_{\min} , but with f_{\max} dependent on the concentration of mecholy (M). To explore these relationships, we proceed from Equation 10 as follows:

The force developed, f , is then given by (assuming additivity)

$$f = f_o A_o + f_r A_r = \frac{f_o KS}{1+K} + \frac{f_r S}{1+K} \quad (12)$$

Because of (eq. 11)

$$f_{\max} = f_o S; \quad f_{\min} = f_r S \quad (13)$$

so that we have

$$f = \frac{K f_{\max} + f_{\min}}{K + 1} \quad (14)$$

From reaction rate theory

$$K = e^{\frac{\Delta F^0}{R} \left(\frac{1}{T} - \frac{1}{T_o} \right)} \quad (15)$$

at T_o , $K = 1$, $A_o = A_r$. If

$$B = \frac{\Delta F^0}{RT_o T} \approx \frac{\Delta F^0}{RT_o^2}, \quad \text{a constant,}$$

the (eq. 14) becomes

$$f = \frac{f_{\max} e^{B(T_o - T)} + f_{\min}}{e^{B(T_o - T)} + 1} \quad (16)$$

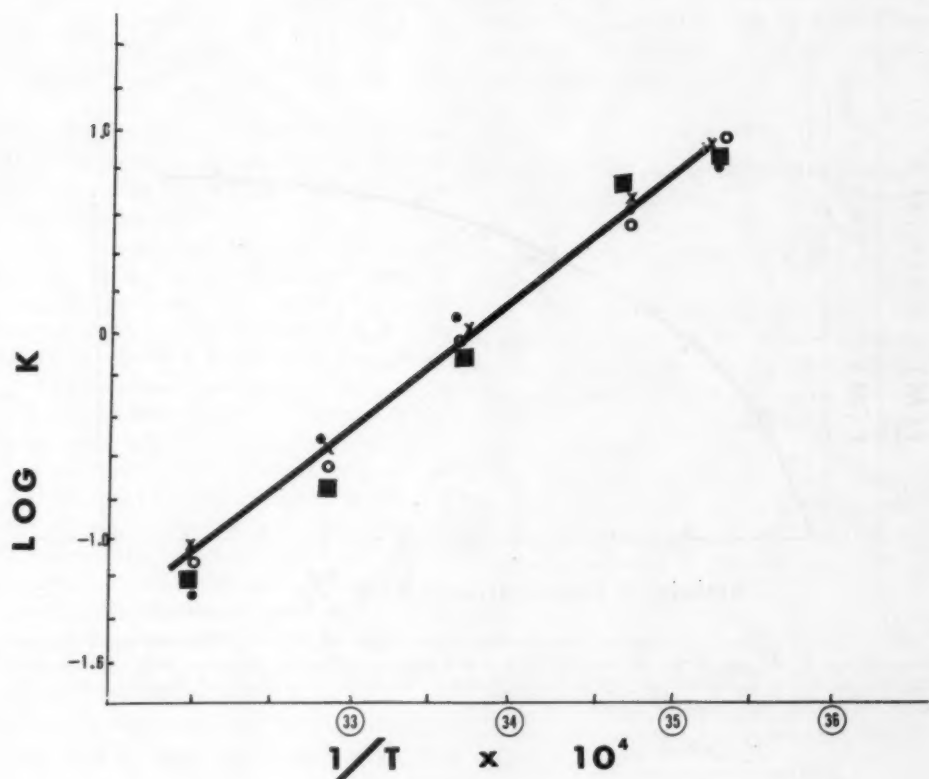


Fig. 1 (Apter). A log-reciprocal plot of K , which equals $\frac{f-f_{\min}}{f_{\max}-f}$ against the reciprocal of the absolute temperature. The line was fitted statistically, using all data from all muscles tested. Each point is the mean of at least five values. Data are depicted as follows: closed circles from dog ciliary muscles; open circles from puppy inferior oblique; squares from cat ciliary muscles; crosses from kitten lateral rectus. Various specimens could not be shown to differ significantly from one another. Essential linearity of the plot is apparent. ΔH is the slope, C , divided by $2.3R$, the gas constant expressed in calories.

Mecholyl effect can be considered in two ways: either as changing the force of each molecule of A_e or by increasing the amount of A_e , which then proceeds in the reaction $A_e \rightleftharpoons A_r$ as before.

CASE 1. Let stimulant M increase f_e by a factor $F(M)$, that is, a given concentration of M makes each molecule of A_e contract by Ff_e instead of by f_e . Then from (eq. 12) the force after stimulation, $f(M)$ will be given by

$$f(M) = \frac{Kf_e F(M)S}{1+K} + \frac{f_r S}{1+K}$$

$$= \frac{Kf_{\max} F(M) + f_{\min}}{1+K} \quad (17)$$

Now $f(M)$ has a "maximum" at low temperatures for $K \gg 1$ (as does f), given by $Kf_{\max} F/(K=1) \rightarrow f_{\max} F = f(M)_{\max}$. (M is the concentration of mecholyl so that $f(M)$ varies with (M) in some way, for example, as already determined according to Equation 11.

$$F = F(M) = f(M)_{\max}/f_{\max} \quad (18)$$

All f_{\max} values are read at 6°C . Hence, from Equations 15, 17, and 18, we have

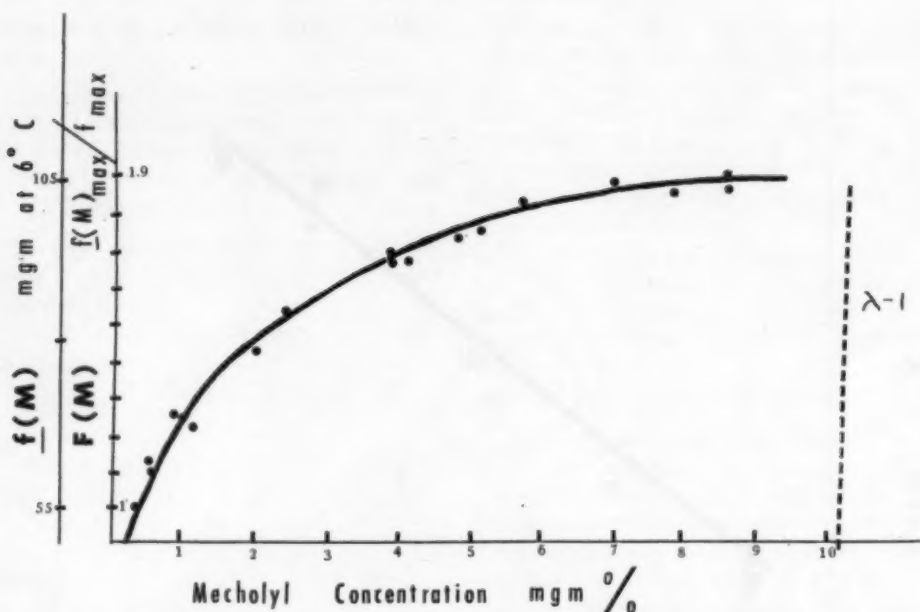
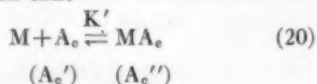


Fig. 2 (Apter). The dose-response curve for the cat iris sphincter at 6.0°C. From the curve may be read $\lambda-1$ and $F(M)$, the factor which converts f at any temperature to the stimulated $f(M)$ corresponding to a particular concentration (M) of mecholyl. Points are actual data corrected to adjust scales. The curve is the fitted form of equation (11).

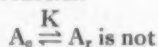
$$f(M) = \frac{f_{\max} e^{B(T_0 - T)} + f_{\min}}{e^{B(T_0 - T)} + 1} \quad (19)$$

Note that $f(0) = f$, and that $f(M)$ is arbitrary, depending on the concentration of M .

CASE 2. Let M react with A_e or activate A_r to A_e' such that



but A_e in the reaction



changed. Then

$$A_e = A_e' + A_e'',$$

where A_e' is the free A_e or activated A_r , and where A_e'' is identical with MA_e , which is that bound to M .

From Equation 20,

$$A_e' = \frac{A_e}{1 + MK'}; \quad A_e'' = \frac{MA_e K'}{1 + MK'} \quad (21)$$

If λf_e is the contractile force of MA_e per mole, then

$$f(M) = f_r A_r + f_e A_e' + \lambda f_e A_e'' = \frac{f_{\max} F(M) + f_{\min}}{1 + K} \quad (22)$$

where $F(M) = \frac{1 + \lambda MK'}{1 + MK'}$. The term $(\lambda - 1)$

is $f(M)_{\infty}$, of Equation 11, (fig. 2). Note that

$$F(M) - 1 = \frac{K' M (\lambda - 1)}{1 + MK'} \quad (22A)$$

and that the equilibrium constant $K' = \frac{1}{M_{50}}$

for M_{50} such that $[F(M_{50}) - 1] = \frac{\lambda - 1}{2}$,

therefore equal to M_{50} of Equation 11, λ being equal to $F(\infty)$. Note also that if the equilibrium constant K' is relatively independent of temperature,

$$f(M) = \frac{f_{\max} \frac{1 + \lambda MK'}{1 + MK'} e^{B(T_0 - T)} + f_{\min}}{e^{B(T_0 - T)} + 1} \quad (23)$$

On the other hand, if λ is less than 1, the M corresponds to an inhibitor. In the case of the intraocular smooth muscles, this could be adrenalin.

These computations are admittedly speculative. Similar computations have been attempted previously: on glycerol extracted muscle at temperatures outside the 6°C to 40°C. range;^{9,16,18} on positive, linear temperature-tension correspondences, making muscle resemble rubber.^{8,10} In neither instance was strict reversibility demonstrated. On the other hand, the present computations are based on data collected with newly devised test procedures demonstrating reversible, reproducible temperature-tension correspondence common to many viable muscles.³ These consistent results make it seem worthwhile to suggest that contractile elements in muscle behave as though in an equilibrium state. Since muscle requires energy for survival, and only surviving muscle has the properties delineated in this paper, a "pseudo"¹⁵ or "dy-

namic"¹² equilibrium must be considered. For this state, the energetic constants computed may be applicable.

SUMMARY

1. Using data describing the temperature-tension correspondence of the intraocular and extraocular muscles of cats and dogs, characteristics of contractile elements are described.

2. The reversibility, sigmoid form and temperature-limits of the tension-temperature relationship are consistent with a hypothetical pseudo or dynamic equilibrium between relaxed and contracted forms of contractile elements.

3. The heat of reaction and entropy change, calculable from the data, are consistent with morphologic and chemical information obtained by other means.

912 South Wood St. (12).

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EFFECT OF INTRALAMELLAR PLASTIC MEMBRANES ON CORNEAL PHYSIOLOGY

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INTRODUCTION

In 1953 Boch and Maumenee¹ attempted to determine if the normal state of deturgescence of the corneal stroma was dependent on constant withdrawal of fluid through the epithelial and endothelial sections of the cornea. To this end they placed thin sheets of polyethylene which extended to the limbus on all sides in the superficial and deep layers of the corneas of rabbits. They observed that the layer of cornea external to the superficial sheet of polyethylene became edematous within four hours but the middle and posterior layers showed no clinical evidence of swelling for forty-eight hours. After this, grey infiltrates developed in the two superficial layers of the cornea and vascularization occurred. On histologic examination of three corneas, forty-eight hours after operation, considerable loss of epithelial and stromal cells was observed in the central portion of the superficial layer of cornea. Fewer cells were lost in the middle stromal layer and the stromal cells in the posterior layer were normal.

When a single sheet of polyethylene, which extended to the limbus on all sides, was placed in the stroma, the same changes occurred in the superficial layer at forty-eight hours. However, if a small disc was placed centrally or if holes were punched in the larger sheet, the superficial stroma and epithelium appeared normal at forty-eight hours.

From these studies they concluded that osmotic forces acting through the epithelium and endothelium as semi-permeable membranes are not solely responsible for the state of deturgescence of the cornea. Also, when the corneal stroma is separated from the endothelium and aqueous by an impermeable membrane that extends to the limbus, the cells superficial to the membrane degenerate. This suggests that diffusion across the cornea to (or from) the aqueous of substance vital to this tissue is essential to survival of the stromal cells.

In recent studies on the regulation of corneal hydration, Langham² concluded that the ability of metabolism to maintain normal hydration is restricted to areas of stroma bordered by intact epithelial and endothelial cells. He observed that corneal swelling produced by removal of the epithelium was completely reversed under the areas of newly developed epithelium even when more than fifty percent of the cornea remained denuded; further, even when ninety percent of the cornea was covered by epithelium, the denuded area remained swollen.

The present study is part of a larger investigation utilizing the techniques of Boch and Maumenee to elucidate the role of the epithelial and endothelial cells in the maintenance of the composition and structure of the stroma (Langham, unpublished). Small, thin, plastic membranes of different physical characteristics were placed between the corneal lamellae of rabbits, cats and monkeys and the reactions of the corneal areas anterior and posterior to the disc were studied (fig. 1).

* From the Wilmer Institute, The Johns Hopkins University and Medical School, Baltimore 5, Maryland. This work was supported in part by funds from the Knights Templar Eye Foundation, Inc.

METHOD

(A): Rabbits of either sex were used. Nembutal[®] was used as a hypnotic and in most cases Thorazine[®] was given. The lids and periorbital skin were cleansed; lid block was performed and a retrobulbar injection with a two percent solution of lidocaine was given. The eye was rinsed with sterile saline solution and a drop of Furacin Ophthalmic liquid[®] was put into the fornix. The globe was proptosed and a sterile eye sheet was draped about it.

An incision slightly longer than the diameter of the membrane to be inserted was made just inside the limbus through the lamellae with a No. 15 Bard Parker blade. With care, a Gill knife was advanced and an intralamellar space was formed. Care was taken not to separate more area than was needed to center the membrane over the pupillary space.

Several methods were used to sterilize the plastic membranes. These included soaking in 1:1000 Quamonium solution, steam sterilization and sterilization in Beta-Propriolactone vapor. The last method was superior for ease of sterilization and allowed the membrane to be formed over a convex surface to acquire the radius of curvature of the cornea and to be trephined to proper size (usually 5 mm.) before sterilizing.

After sterilization, the membrane was rinsed in sterile saline solution and then carefully advanced in the intralamellar space to the center of the cornea. The incision was closed with 7-0 black silk sutures, and the eye was allowed to fall back into the socket (fig. 2). Furacin drops were applied.

In four eyes, two membranes were placed

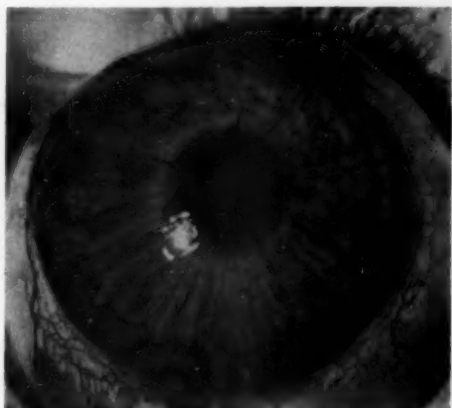


Fig. 2 (Knowles). Rabbit's eye one day after insertion of a 5 mm intralamellar polypropylene membrane. No. 67 L.E.

into the cornea concentric with each other, so as to isolate a segment of stroma from the limiting layers of the cornea. Two incisions were made at separate sites on the limbus and to different depths into the stroma. An intralamellar space was formed at each depth and the membranes were inserted in the same manner as for a single membrane.

Membranes were placed into 62 rabbit eyes. Thirteen of these became infected. Twenty-six of the 49 remaining eyes were observed closely, some for as long as five months (table I). The others were fixed at intervals for histologic study or were used for ancillary experiments.

The thicknesses of the corneas were measured preoperatively with a corneal pachometer used on the illuminating arm of a Haag-Streit slit lamp.³ Measurements were also made immediately after operation, at three hours, at one and two days, and then at intervals of one or more days.

Membranes used were: polyethylene, thickness 12 micra and impermeable to fluid; polyvinylidene, thickness 12 micra and considered impermeable* to water vapor and



Fig. 1 (Knowles). Schematic representation of cornea with an intralamellar plastic membrane.

* Calculations for a membrane 2.2 cm.² (the area of the rabbit's cornea) would give a maximum water vapor transmission at 32°C and 90 percent relative humidity of approximately 1.2 mg./24 hours for both

TABLE I
EFFECT OF INTRALAMELLAR PLASTIC MEMBRANES ON RABBIT CORNEAS*

Rabbit	Size of Membrane (mm.)	Comment	Day Crater Began	Observ. Period (days)
1	5		11	11
2	7	(Vascularization noted at 13 d.)	21	29
4	6.5	(Vascularization noted at 12 d.)	20	28
6	5		14	18
8	5	Two membranes inserted, one under epithelium, one over Descemet's membrane. Degeneration occurred in surface layer.	16	24
10	5	Hypopion at 4-6 d.; cleared on antibiotic drops.	16	29
18	5	Anterior stromal density began day 14.	29	32
19	5	Haze of stroma developed on day 11, suggesting degeneration of this area would soon occur.		12
20	5	Haze of stroma developed on day 12.		12
21	5	Plastic very close to Descemet's membrane.	34	59
24	5	(Corneal endothelium removed day 3. Deturgescence complete on day 18).	32	40
32	5	(Endothelium partially removed on day 21. Deturgescence complete on day 28).	31	87
37	5	(Endothelium removed on day 0. Deturgescence complete on day 20).	25	45
43	5	(Endothelium removed day 0; Deturg. day 7).	13	68
48	5	(Vascularization noted at 26 days).	13	26
49	5	Infection noted at 48 days.	25	48
51	5	Plastic very close to Descemet's membrane.	14	47
52	5		23	53
53	5		14	53
54R	5	Dense haze of central cornea present on day 30. Membrane removed & cornea became crystal clear.		175
54L	5	Dense haze of central cornea present on day 30. Membrane removed & cornea became crystal clear.		175
58L	5	Collodion membrane of 5A porosity. Degeneration of stroma did not deepen to the membrane.	41	62
58R	5	Tarsorrhaphy maintained for 3 weeks postoperatively. Degeneration 3 mm. diameter, did not deepen to the membrane.	49	85
61	8.5	Slight central haze only.		105
67R	5	Two membranes inserted, one under epithelium, one over Descemet's membrane. Degeneration occurred in surface layer.	9	13
67L	5	Disc removed at 13 d. Crater filled with epithelium and remained clear.	9	85

* Type of Membrane used: No. 1-10: Polyethylene.
No. 18-43, 51-54: Polyvinylidene.
No. 48, 49, 58R-67: Polypropylene.
No. 58L: Collodion.

gas; polypropylene, thickness 6 micra and considered impermeable to water vapor, but permeable to carbon dioxide and oxygen;

polyvinylidene and polypropylene. This calculation, based on the maximum transmission, would be high for the conditions existing in the cornea. Calculations for oxygen and carbon dioxide transmission at 23°C are:

Polyvinylidene	Polypropylene
Oxygen:	
3.3 microlitres/24 hrs	360 microlitres/24 hrs.
Carbon dioxide:	
12.5	910

Calculations above are based on information kindly supplied by the Dow Chemical Company.

Visking's cellulose acetate dialysis tubing with a thickness of 19 micra and a porosity of 24 A.; a dried collodion membrane with an estimated thickness of 6 to 8 micra and a porosity of approximately 5 A.⁴

(B): Cats and monkeys were anesthetized with Nembutal and prepared in much the same manner as the rabbits. Lateral canthotomy was done before the lids were retracted. At all times during the operation, the cornea was kept moist with sterile saline solution (figs. 3 and 4).

Eyes for histologic study were fixed in formalin or preferably Zenker's solution.

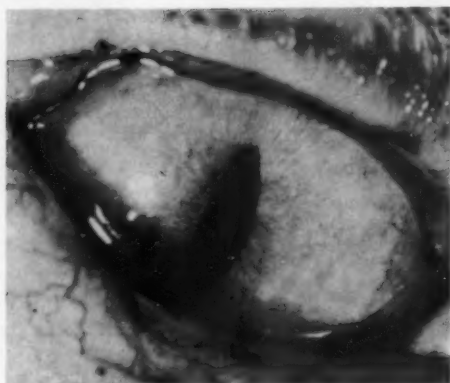


Fig. 3 (Knowles). Cat's eye one day after insertion of a 6.5 mm polypropylene membrane. No. 1 R.E.

The intact eye was fixed and sectioned; distortion resulted when the cornea was removed at the limbus to be fixed and sectioned. Haematoxylin and eosin were used for staining. Schiff's periodic acid staining reaction, colloidal iron, Verhoeff's stain and Weigert's resorcin-fuchsin stain were also used.

RESULTS

Corneal changes external to the plastic membrane were observed in all 26 rabbit eyes (table I). In 21 of these degeneration

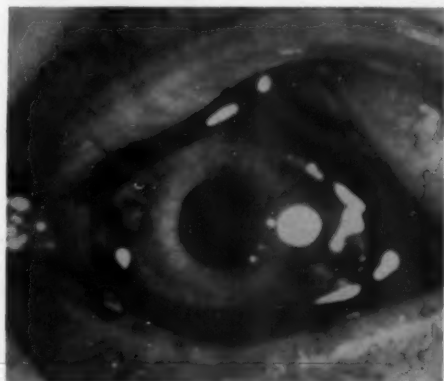


Fig. 4 (Knowles). Monkey's eye three days after insertion of a 5 mm polypropylene membrane. No. 2 L.E. (The appearance of the cornea did not change during a 3-months period of observation. It remained crystal clear and ophthalmoscopic examination revealed no optical distortion.)

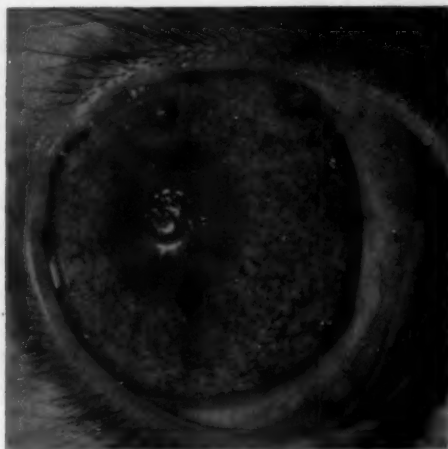


Fig. 5 (Knowles). 18 days after insertion of a polyethylene membrane. (Polyethylene is not as clear as other plastics used.) No. 6 R.E.

developed with disappearance of substantia propria immediately over the membrane within nine to thirty-four days (fig. 5). Two of the 26 were examined for histologic changes at ten and eleven days after insertion of the membranes. In each of these the cornea over the membrane had developed a dense haze, and it was anticipated that the degeneration and disappearance of the stroma was about to occur. Two corneas were reoperated upon thirty days after insertion of the membranes. In each of these a haze had developed in the stroma anterior



Fig. 6 (Knowles). Histologic appearance of a crater in a formative stage, 32 days after insertion of the membrane. No. 18 R.E.

TABLE II
EFFECT OF INTRALAMELLAR PLASTIC MEMBRANES ON CAT CORNEAS

Cat	Size of Membrane (mm.)	Result	Interval	Observation Period (days)
1	6.5	Very slight haze centered over the membrane	At 49 days	67
2	6.5	Crater developed over the membrane (a small hypopion was present on day 8, following a second operation, an intraocular procedure.)	9	9
3	6.5	Haze over the membrane Crater	20 29	29
4	6.5	Endothelium removed Deturgesced Crystal clear	7 14 28	29 (Died of an infection from a fight.)
5	6 Methyl methacrylate* 0.12 mm. thick	Areas of density at the margins Eroding through the anterior cornea Vascularizing	4 20 26	26

* Provided through the courtesy of Obrig laboratories, Florida.

to the membrane, but the defect had not developed. The membranes were removed. In one of the 26 corneas an 8.5 mm instead of the usual 5 mm membrane had been inserted and a defect did not form by the fifty-fifth day.

The defect formed by the degeneration and disappearance of substantia propria was well characterized. It began as a small concavity on the epithelial surface of the cornea and rapidly deepened to the center of the plastic membrane. On microscopic examination of the cornea at an early stage of degeneration of the stroma, epithelial hyperplasia was seen to fill the defect partially.

Few inflammatory cells were seen in the stroma (fig. 6). The small, well circumscribed defect, or crater, enlarged in diameter daily for two to ten days to a maximum of 3.5 mm and in no case extended to or beyond the border of the membrane. Posterior to the membrane there was no corneal change.

Corneal changes in the cat eyes (table II) were more variable in uniformity and time of occurrence than were those in the rabbit eyes. Two of these formed a crater over the membrane, one at nine and one at twenty-nine days; in one a slight haze developed by the forty-ninth day. One cornea became

TABLE III
EFFECT OF INTRALAMELLAR PLASTIC MEMBRANES ON MONKEY CORNEAS

Monkey	Size of Membrane Inserted (mm.)	Result	Interval	Observation Period (days)
1	5	Small point of opacity Vascular twig approached the membrane Cornea clear	At 8 days 54 97	97
2	5	Crystal clear	90	90
3	6.5*	Turgesced Turgesced and vascularized	1 14	44
4	5	Pin-point opacity Cornea clear	9 44	44

* The cornea measured 10 mm. horizontally.

infected and one extruded the membrane. (This was a 0.12 mm thick methyl methacrylate disc). There was no clinical or histologic evidence of change in the tissue internal to the disc.

The corneas of three monkey eyes, into which had been placed polypropylene membranes 5 mm in diameter, remained clear for as long as three months, the duration of observation in two (table III). There was no change in them other than slight accentuation of the stromal pattern in front of the membrane as seen by slit lamp examination. A small opacity developed over the periphery of the membrane in two corneas in the early postoperative period and a single blood vessel extended to the membrane in one of these. The third was crystal clear without a defect. A fourth monkey eye received a 6.5 mm membrane, which it did not tolerate. This cornea turgescd and subsequently vascularized.

On microscopic examination of the corneas in which craters developed, sharply localized absence of the substantia propria external to the plastic membrane was observed (fig. 7). In its place a downgrowth and hyperplasia of epithelial cells eighteen to thirty cells deep was found. The normal columnar basal cell layer was absent; flattened cells formed both surfaces of the epithelial downgrowth, and midway between, the cells developed cuboidal characteristics. The epithelial downgrowth extended peripherally over the plastic membrane. The stroma around the crater was not strikingly abnormal. Inflammatory cells

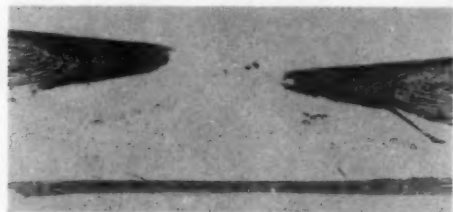


Fig. 7 (Knowles). Histologic appearance of corneal crater 35 days after insertion of the membrane. No. 51 R.E.

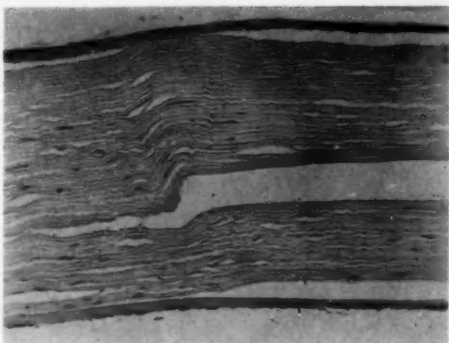


Fig. 8 (Knowles). Histologic appearance at the margin of the membrane in a 48-hour section. No. 46 R.E.

were sparse and keratocytes were present. Tissue and cells internal to the membrane appeared to be normal.

The course of events after insertion of the membranes was closely followed. Transient increases or decreases in corneal thickness of 5 to 15 percent were measured. These changes were associated with excessive use of saline solution during insertion of the membrane or with drying of the cornea, respectively. The thickness of some corneas did not change during the entire period of observation.

Eleven rabbit eyes (two of these controls) were examined microscopically forty-eight hours after insertion of the membranes. The experimental eyes showed epithelial thinning over the membrane. The corneas that had not been protected by taping the lids closed during the immediate postoperative period showed apparent absence or strikingly diminished number of keratocytes anterior to the membrane. The keratocytes were present directly adjacent where the cornea was intact (fig. 8). These changes were not seen in control corneas.

At eight to fifteen days, on slit lamp examination, a haze of the stroma external to the membrane was observed. This haze became apparent on hand light examination before formation of the craters. It was seen to lie directly over the membrane with a

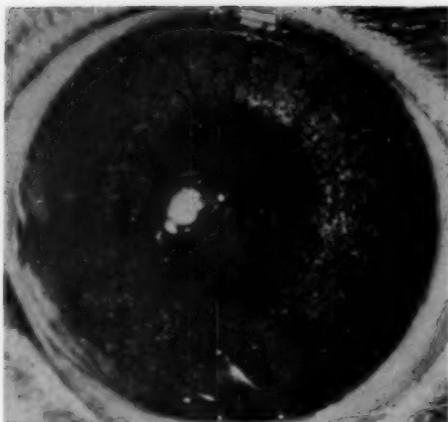


Fig. 9 (Knowles). Rabbit's eye 28 days after insertion of a 5 mm polyvinylidene membrane. The membrane was perforated by a central 1.5 mm. hole and several peripheral pinholes. No. 25 R.E.

border one half to one millimeter within the margin of the membrane. Development of the craters was not accompanied by discernable change either behind the membrane or in the rest of the cornea.

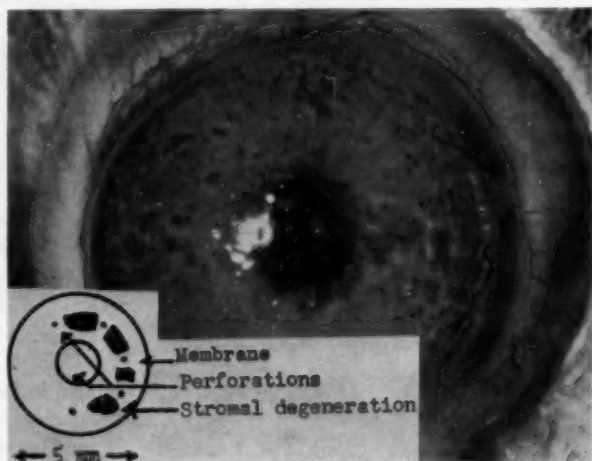
Membranes perforated by a central 1.5 mm hole and seven or eight pinholes peripherally were inserted intralamellarily into the corneas of nine eyes to determine if similar changes in the corneas would still occur (fig.

9). In no instance did a crater form. The period of observation was eight months. The cornea over the central perforation stayed crystal clear. However, at eleven to seventeen days, a haze of the stroma could be seen on slit lamp examination over the plastic, but not over the central hole. This haze increased in density so that by fourteen to thirty-five days a sharply localized ring of opacity broken by small clear areas (over the pinholes) lay over the membrane (fig. 10). The areas of opacity were extremely discrete. They occurred where the cornea was disrupted even though less than 1 mm away in the plane of the cornea, the vertical integrity was intact. Degeneration and disappearance of the substantia propria external to the membrane did occur in areas of the ring of stromal density.

DISCUSSION

Without exception, in rabbit eyes, a degenerative process occurred external to the plastic membrane. This was not believed to be a toxic reaction to the membrane, as it occurred with each of three different plastic materials. The absence of inflammation and the sharp localization of the defect external to the membrane without change internal to it are also evidences against a toxic reaction.

Fig. 10 (Knowles). Perforated membrane (with schematic insert), 26 days after insertion of the membrane. There are discrete areas of stromal degeneration centered over the membrane. The cornea is crystal clear over the perforations. (Shadows incurred with photography.) No. 52 R.E.



This suggested interference by the membrane with a physiologic mechanism necessary to the vitality of this tissue. It also suggested that the relationship between this layer of cornea, lying anterior to the membrane, with the limbus, the surrounding intact cornea and the surface film was not adequate to maintain vitality.

The size of the membrane could be small; when it was only 2 mm. across, the degenerative process still occurred (fig. 10). The discreteness of the lesion over the membrane suggested that there must be a need for a direct interrelationship between a segment of the anterior layer of the stroma with the cornea lying directly underneath.

The depth at which a membrane was placed in the stroma, superficial, deep next to Descemet's membrane, did not appear to influence the time at which the crater would develop.

This process in which the stroma overlying an intralamellar membrane degenerates and disappears is unique. The tissue behind the membrane appears to be maintained quite satisfactorily. However, in front of the membrane it disappears even though surrounded by normal, intact cornea. The crater might be the result of one of three causes: 1) lack of vital substance from the aqueous humour, 2) accumulation of a toxic substance, 3) selective drying of the cornea lying anterior to the plastic.

The time factor was extremely variable and the process did not occur in all species. This suggests something more than simple interference with normal nutrition.

Diffusion: It might be supposed that there would be diffusion within the stroma, in the plane of the cornea, of fluid, vital nutrients or toxic products sufficient to maintain the nourishment of the cornea. We know that diffusion does take place in the plane of the cornea as well as across it.⁸⁻⁹ However, the resistance to diffusion of Na²⁴ in the stroma from the limbus is eight times its value in free solution.⁶ Maurice¹⁰ recently suggested

from studies on diffusion of fluorescein in the stroma that there is no appreciable flow in the plane of the cornea. In corneas with an intralamellar plastic barrier, in which the tissue degenerates over the membrane, there must be strong resistance to the net movement of fluid in the plane of the cornea. The intralamellar membrane greatly interfered with the diffusion of fluorescein in the stroma. Fluorescein introduced through a small epithelial defect over the plastic membrane was found to be present in the stroma, over the membrane 36 hours later. Initially, it would diffuse laterally and posteriorly; at no time was it seen behind the membrane. A similar normal cornea cleared the dye in six to eight hours.

The plastic membrane also interfered with ingress of fluid from the aqueous humour into corneas from which the endothelium was removed. This observation was made in cats and rabbits. The stroma internal and peripheral to the membrane would rapidly swell. The area over the membrane would maintain its thickness for as long as 24 hours at which time edema would be seen to extend into it from the surrounding stroma. After several days the cornea would regain its normal thickness. The area over the membrane deturgescenced concurrently with the rest of the stroma.

Oxygen: It would be extremely unlikely that the pathogenesis of the degeneration of stroma over a plastic membrane would be related to interference with oxygen diffusion by the membrane. Our knowledge of the metabolism of oxygen by the cornea¹¹ would make this improbable, but the fact that the crater develops with polypropylene which is permeable to oxygen in quantities sufficient to meet the demands of the cornea (300 microlitres/24 hours¹¹) as well as with polyvinylidene, which is impermeable to oxygen, would rule out any direct association with oxygen metabolism.

Mucopolysaccharide: There does not appear to be an early change or breakdown

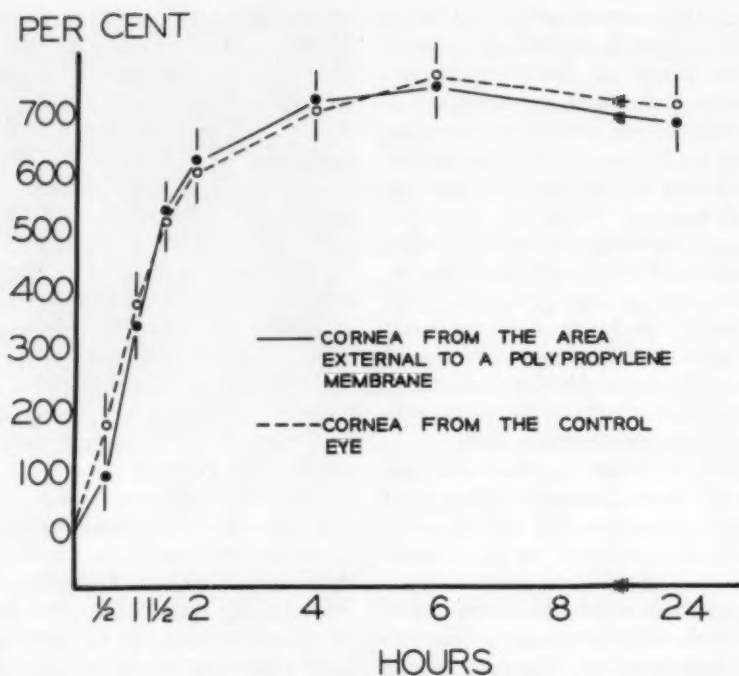


Fig. 11 (Knowles). Comparison of the swelling of cornea removed from the area external to a polypropylene membrane with cornea from the other eye. Cornea was removed six days after insertion of the membrane and immersed in distilled water at 4°C.

of the mucopolysaccharide complex as determined by corneal swelling experiments designed directly to reflect the mucopolysaccharide content of the area of cornea over the membrane (fig. 11).

Drying: The intramellar plastic membrane is a barrier to diffusion of fluid across the stroma for the small area of cornea being studied. If there is great resistance to the flow of fluid in the plane of the cornea, selective desiccation of stroma external to the membrane may occur. Drying of the cornea overlying the membrane is suggested in sections of corneas at 48 hours by epithelial thinning directly over the membrane. Thinning was greatest in corneas not protected by taping the lids closed in the immediate postoperative period. The cornea would stain over the membrane two to three hours after the operation when the lids were not taped

closed to prevent drying. Tarrorrhaphy was done on the lids of three rabbit eyes and the lids were kept closed for three weeks postoperatively to prevent drying. Two of these eyes became infected. The third eye did not develop a crater until the forty-ninth day, which was considerably longer than the usual nine to 34 days interim.

Prevention of drying may be related to the frequency of spontaneous blinking of the eyelids. The frequency of blinking of the rabbit's and the cat's eyelids is slow, whereas it is rapid with the primate's.¹² The rapidity with which the primate blinks, thereby renewing the tear film over the cornea, may prevent selective desiccation of the corneas in which the membranes were placed.

Size of the Plastic Membrane: The size of the membrane is important. A full corneal

intralamellar membrane, limbus to limbus will result in vascularization beginning in 48 hours.¹ Membranes, 5 mm in diameter, placed in rabbit and monkey eyes did not cause vascularization, nor did 6.5 mm membranes in cat eyes. A 6.5 mm membrane placed in a monkey's cornea, which had a horizontal diameter of 10 mm., resulted in postoperative corneal edema and vascularization of the cornea beginning on the fourteenth day. Two of three 8.5 mm membranes placed in rabbit corneas resulted in vascularization; the third was extremely well tolerated. The cornea remained crystal clear for 49 days at which time a slight haze of the stroma over the center of the membrane developed. Progression of this stromal change had not occurred by the third month postoperatively. The tolerance of the cornea to the 8.5 mm membrane was in contrast with the stromal degeneration that occurred over the 5 mm membranes within nine to 34 days.

All membranes used in cat corneas were 6.5 mm in diameter (table II).

Porosity: Visking's cellulose acetate, which has a porosity of 24 Å., was placed into 6 corneas; 5 of the 6 became infected. This is in accord with the observations of Day¹³ who reported the toxicity of cellulose acetate upon the cornea. However, one cornea was relatively resistant to this toxic effect and other than development of transient, mild turgescence in the postoperative period, tolerated the membrane for 135 days, after which it began to extrude. There was no crater or any central area of haze over the membrane, which suggested that the vital physiologic relationship with the underlying tissue was maintained through porous connections of 24 Å. diameter. With a thin, collodion membrane of porosity 5 Å., the crater was delayed, but was not prevented.

Reversibility: If the membrane was removed just before formation of the crater, even though a haze or density of the central cornea had appeared, the process was

halted. The cornea became crystal clear. Some optical aberration was present, presumably from damaging the lamellar structure. In a cornea in which the membrane was removed after the crater had formed the defect in the stroma remained, but completely filled with epithelium. The cornea was clear, but the area filled with epithelium appeared to have a different index of refraction than the surrounding stroma. During the two and one-half months of observation this defect was not replaced with collagen tissue.

CONCLUSIONS

1. Plastic membranes were inserted intralamellarly into corneas of rabbit, cat and monkey eyes without subsequent scarring or vascularization of the corneas.

2. Degeneration of the substantia propria anterior to an intralamellar plastic membrane occurred in rabbit and cat corneas, but monkey corneas remained clear.

3. The degeneration of the stroma in rabbit and cat eyes is very discrete and occurs where the integrity across the cornea is interrupted by less than 2 mm. This suggests strong resistance to net movement of fluid in the plane of the cornea.

4. Intralamellar membranes interfered with diffusion of fluid across the part of the cornea in which the membrane was placed.

5. Toxicity of the plastic membranes upon the cornea, interference with diffusion of fluids and gaseous metabolites across the cornea, breakdown of mucopolysaccharide complexes, and selective desiccation of the stroma external to the intralamellar membrane were discussed as possible causes of degeneration of the substantia propria in rabbit and cat corneas. It is concluded that desiccation of the substantia propria anterior to the membrane occurred and that diffusion in the plane of the cornea is insufficient to maintain corneal hydration. In the primate eyes the rapid spontaneous blinking of the lids over the corneas will maintain a tear

film that will prevent the desiccation and degeneration of substantia propria anterior to the membrane.

*Ochsner Foundation Hospital,
New Orleans (21), La.*

ACKNOWLEDGMENT

I wish to express my sincere thanks to Dr. A. E. Maumenee and to Dr. M. E. Langham, for their interest and advice.

ADDENDUM

Since this paper was written, we put a 5½ mm polypropylene disc into the cornea of a 73 year old man, a patient of Dr. Robert Schimek, at the Ochsner Foundation Hospital. This patient was aphakic, had advanced Fuch's dystrophy and had received bilateral penetrating grafts five and three years before. The corneas were vascularized, the stroma was edematous and nearly opaque and there was epithelial edema and blebs. Vision was counting fingers at five inches. The disc was placed in the stroma and centered within the margin of the scar of the 8 mm

graft. Vessels leading into the cornea on the side of the incision were cauterized. The membrane did not incite increased vascularization or inflammation, nor was there any change in the edema or transparency of the cornea. The surgical incision, which was made within the margin of the scar of the penetrating graft did not heal and 27 days after the operation, a 9 mm lamellar keratoplasty with a glycerine preserved cornea was done. A 3 mm plastic disc was placed between the graft and the recipient cornea. The graft healed well. Vessels extended into it, but did not advance to the area over the plastic. The graft became edematous, but could be 40 percent deturgescenced in the area over the plastic with warm air from a hair dryer. The cornea was constantly bathed in a pool of tears and the warm air from the dryer stimulated the flow of tears even more. The eye was followed for a ten week period, during which time the membrane did not appear to incite inflammation or vascularization.

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A TECHNIQUE FOR EXTRAOCULAR ELECTROMYOGRAPHY IN CHRONIC ANIMALS*

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In the past decade there has been increasing application of electromyographic techniques for study of the function of extraocular muscles. These investigations are of two types: study of normal physiology and functions, and secondly, study of the muscles in diseased states, particularly neurologic or neuromuscular abnormalities. Based on existing knowledge of electromyographic changes in disease, clinical diagnoses are made, or more often substantiated, by the recordings from a particular patient. However, without subsequent surgical exploration or autopsy findings, it is impossible to definitely correlate an electromyography recording with a specific disease or lesion.

With this in mind, it would be advantageous to correlate electromyographic recordings from the extraocular muscles with known lesions in experimental animals. To achieve a satisfactory correlation, the animal preparation should be otherwise physiologic; that is living and alert, neither narcotized nor anesthetized. Since it is not practical to apply the usual clinical method of transconjunctival insertion of needle electrodes in such an animal, another approach is necessary. The purpose of this paper is to describe a technique for recording electromyograms from the extraocular muscles of the cat in such a way as to fulfill the criteria described.

TECHNIQUE

The surgical approach is through the frontal sinus. A v-shaped incision is made above the brow with the apex superiorly

(fig. 1). The wedge-shaped skin flap is reflected down over the eye and the portion of the frontal bone covering the frontal sinus is removed with trephine and rongeurs. The floor of the sinus, the roof of the orbit, is easily removed with rongeurs and after careful dissection of the overlying loose fat, the superior rectus and oblique muscles are easily identified (fig. 2).

The medial and lateral rectus muscles may be brought into the field by rotation of the globe and traction with a muscle hook.

The electrodes used consist of 38-gauge enamel insulated, nichrome wires which are paired and inserted into the muscle belly and around the tendon respectively (fig. 3). Before insertion, the wire for the muscle belly is denuded of insulation at one spot for a length of about 1.0 mm. The wire is held by curved forceps near its distal end and with a quick spearing motion can be made to pierce the muscle sheath without bending. It is then passed through the muscle until the bared area lies within the belly. The portion of wire emerging from the muscle is then looped around the portion that enters the muscle to provide fixation of the bared area.

The second wire is bared of insulation for approximately 1 cm. and wrapped repeatedly around the tendon near its insertion. This wrapping may be firm without constricting the tendon. A short distance from the muscle the nichrome wires connect with common, rubber insulated, electric wires which are passed upward out of the orbit and frontal sinus and led back beneath the scalp to emerge through a skin puncture in the posterior cervical region. The ends of these wires may be allowed to dangle freely without damage to themselves or to the animal (fig. 4). This procedure is repeated for each

* From the Department of Ophthalmology, College of Medicine, State University of Iowa, Iowa City, Iowa. Presented at the Midwestern Section of the Association for Research in Ophthalmology, April 23, 1960, Indianapolis, Indiana.



Fig. 1 (Stuart). The cat head as seen from above. (Left) A V-shaped skin flap is made and reflected downward. Fig. 2 (Right) The roof of the frontal sinus and roof of the orbit have been removed. The superior rectus muscle is seen on the muscle hook with the globe beneath.

Fig. 3 (Stuart). The electrodes consist of 38-gauge enamel insulated nichrome wire soldered to electric wire. One wire passes through the muscle belly and the other around the tendon.

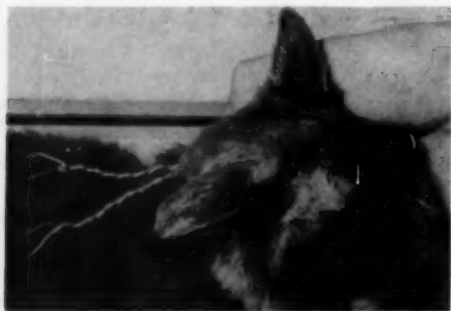
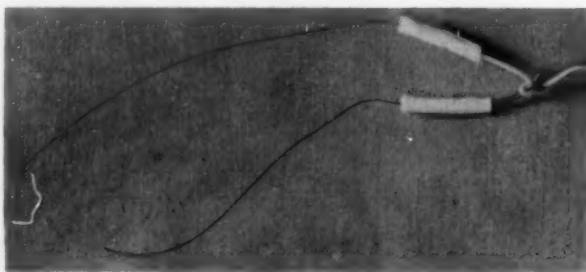


Fig. 4 (Stuart). The cat after insertion of electrodes. The electric wires pass up out of the orbit, beneath the scalp and emerge through a cervical skin puncture.

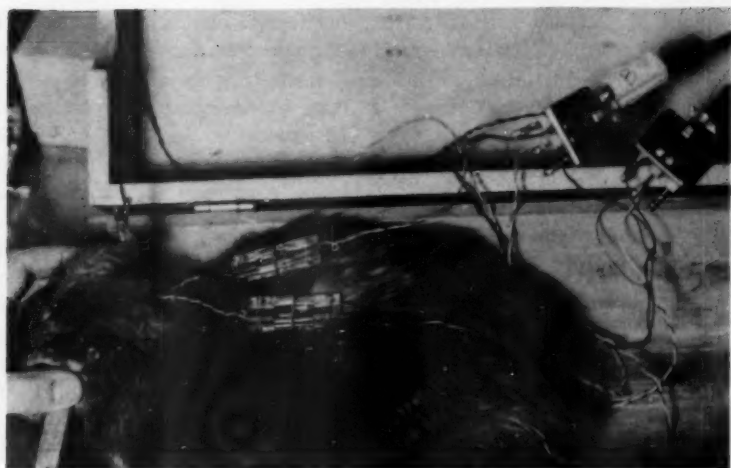


Fig. 5 (Stuart). Television antenna plugs may be used to facilitate connection to the oscillograph leads which are seen on the right. A ground wire from the tip of the ear goes to each lead.

muscle and the skin flap then replaced to cover the opening.

It has been found that replacement of the orbital roof is unnecessary and the sinus area is filled with fibrous tissue within a few weeks. Care must be taken to insure

that the nichrome wire portion of the system is not lengthy, since the insulation will rub off on the bony edges of the defect. The free external ends of the electrodes may be quickly and repeatedly connected to oscillograph leads at any time without further

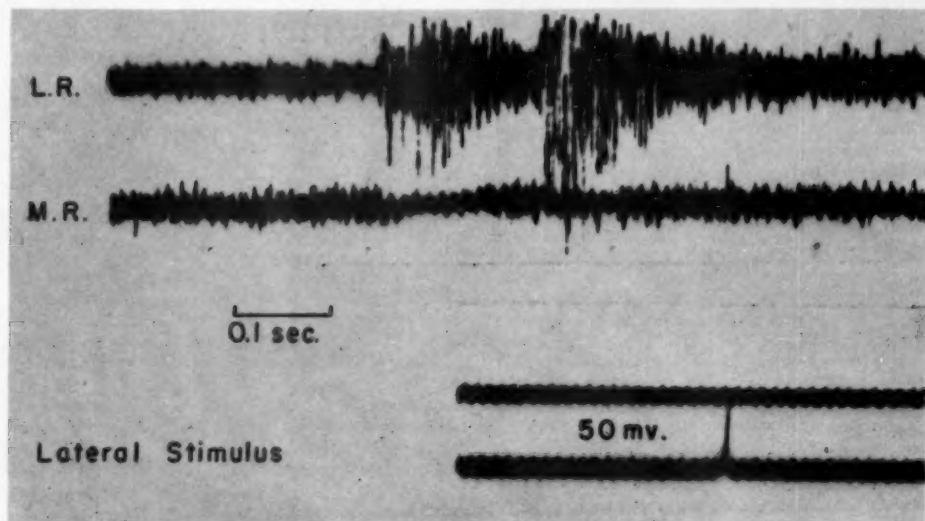


Fig. 6 (Stuart). Electromyogram from the living cat showing saccadic movement laterally on sudden stimulus. Rotation of the head toward the stimulus prevents sustained versions. Signal is 50 microvolts.

manipulation of the animal (fig. 5). Recordings are made with a Grass Kymograph Camera at film speeds of 100 or 250 mm. per second.

COMMENT

A problem which arises in the study of the cat is its natural preference to move the head, not the eyes, when changing direction of gaze. This may be overcome by allowing the animal to rest comfortably in a cat box in a quiet, darkened room and then suddenly presenting a stimulus in the field of gaze desired. A toy rubber squeak mouse does nicely as the stimulus. With sudden stimulation, saccadic eye movement temporarily precedes the head movement and is reflected in the electromyograph (fig. 6). For those who have a plethora of time and assistants, a conditioning method for training eye movements in cats has recently been presented by Richardson and Davis.¹

Subsequent exploration of the electrodes two months after insertion has shown them to be intact and in position. Sometimes the muscle is found to be attached by scar tissue to the globe or orbital wall at or near

the area of electrode insertion. It is possible that such scarring may result in immobilization of the globe and subsequent recordings may be of isometric contraction. Breinin² has found in his acute preparations that isometric contraction provides the most reliable recordings. I am uncertain whether this appreciably changes the desired physiologic state in this chronic preparation. It would seem to be similar to the situation which may exist after clinical muscle surgery.

Further studies are now planned to determine if this method will demonstrate consistent electromyography changes following experimental lesions in the brain stem, peripheral nerves and extraocular muscles.

SUMMARY

A technique for recording electrical potentials from the extraocular muscles of chronic animals is presented. Using this technique, electromyograms have been recorded which demonstrate characteristic resting and contraction patterns.

State University of Iowa.

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PROCEEDINGS

OF THE

ASSOCIATION FOR RESEARCH IN OPHTHALMOLOGY, INC.

OFFICERS 1960

James H. Allen
V. Everett Kinsey
Harold F. Falls
Alson E. Braley
John E. Harris
Irving H. Leopold
Lorand V. Johnson

Lorand V. Johnson

BOARD OF TRUSTEES

Chairman

Secretary
Assistant Secretary
Treasurer

OFFICERS 1961

V. Everett Kinsey
Harold F. Falls
Alson E. Braley
John E. Harris
Irving H. Leopold
Bernard Becker
Monte G. Holland
Robert Moses
A. Ryan Irvine, Jr.

BUSINESS SESSION

June 14, 1960

The annual business session of the Association for Research in Ophthalmology, held on Wednesday, June 14, 1960 at the Eden Roc Hotel, Miami Beach, Florida, convened at 11:30 a.m., Dr. James H. Allen, Chairman, presiding.

CHAIRMAN ALLEN: I now declare the business meeting of the Association for Research in Ophthalmology open.

The first order of business will be the report of the Secretary-Treasurer.

SECRETARY LORAND JOHNSON: Eighty-eight new members were approved for full membership by the Board of Trustees at this meeting. The total membership is now 1,428. The May Supplement with membership statistics of 1959 has been available to the membership.

The Treasurer's report is as follows: An anonymous gift of \$1,000 to the Friedenwald Fund is announced to the membership. The bank balance as of June 8 was \$5,181.49. There are no major outstanding obligations. The financial report for 1959 and the auditor's report were published in the May Supplement and have been available to the membership.

CHAIRMAN ALLEN: Thank you, Dr. Johnson.

The Auditing Committee consisting of Dr. George M. Haik of New Orleans and Dr. Werner K. Noell of Buffalo report that the books appear to be in satisfactory order, and they agree with the commercial auditor's report that the Treasurer has kept an excellent set of books.

I would like to add this comment at this time: The Chairman and the Board of Trustees feel that the Secretary-Treasurer has done an outstanding job during his tenure of office in increasing the

efficiency of the office of the Secretary-Treasurer and in increasing the membership of the Association for Research in Ophthalmology. In the six years during which he has been Secretary-Treasurer, the membership of the Association has doubled.

Thank you very much, Dr. Johnson. (Applause)

DR. BEDELL: Mr. Chairman, I move you, sir, that a record of your commendation be passed by the Association and entered upon the minutes as thanks to our Secretary-Treasurer for his efficiency.

VOICE: Second the motion.

CHAIRMAN ALLEN: Thank you very much, Dr. Bedell, and thank you for the second.

(The motion was put to a vote and was carried unanimously.)

CHAIRMAN ALLEN: At this time I would like to report that the Trustees of the Association have been busy considering a number of things in the last few days, and they wish to report to you that in the future, if the American Medical Association is willing to continue to have joint meetings, we recommend that the meetings of the Association for Research in Ophthalmology be limited to three half-day programs in order to enable our membership to attend more of the scientific sessions of other sections, and to get more out of the scientific exhibits.

In lieu of this time which we are sacrificing, we feel it is necessary for us to provide other time for research papers and an outlet for reports of research work, which is increasing, and for that reason we hope to be able to expand the Mid-winter National Meeting of the Association for Research in Ophthalmology in the future.

Furthermore, in view of the expanding activities of the Association for Research in Ophthalmology, the Trustees have received a set of proposed amendments to the Constitution and Bylaws which,

in brief, propose a separation of the office of Secretary-Treasurer into two offices—an office of Secretary and an office of Treasurer—and also provide for the election of an Assistant Secretary.

The changes that are necessary in order to accomplish this are covered in Articles 4, 5, 6, 7, 8, 9 and 10 of the Bylaws. If it is the desire of the organization, I shall read the changes that are recommended, but I can say that these have been unanimously approved by the Board of Trustees.

What is your desire on this aspect of the proposed changes in the Constitution and Bylaws?

DR. BEDELL: I move they be accepted without reading.

DR. VON SALLMANN: Second the motion.

DR. HERMANN M. BURIAN: One question, Mr. Chairman. Are there any provisions in the proposed Bylaws about how such constitutional changes are to be made? They are usually presented three months ahead, or something like that. Isn't that the usual procedure? This is a very important step.

CHAIRMAN ALLEN: Yes. The provision for amendments requires that the amendments in writing, signed by three members, be circulated to the Board of Trustees one month before the meeting. They then are presented to and discussed by the Board of Trustees and approved or disapproved. Whether they are approved or disapproved by the Board of Trustees, they then must be presented to the Association, where they are accepted or rejected.

The proposed changes were received and distributed to the Trustees one month in advance of the meeting. The Trustees have discussed them. The Board of Trustees recommends that the proposed changes be passed.

Any further questions or discussion?

(The motion was put to a vote and was carried unanimously.)

CHAIRMAN ALLEN: In addition, there is a proposed change in the Bylaws in Article 3 which has become necessary, also, because of the expanded activities of the Association for Research in Ophthalmology, which increases the dues of the Active members from \$10 per year to \$15 per year and the dues of the Educational members from \$3 to \$5 per year. If you wish the Article read, I shall be glad to read it, but that is the essential change. The Trustees have recommended it.

VOICE: May I ask why it is necessary to do this?

CHAIRMAN ALLEN: It is necessary to do this because of increasing expenses of maintenance of the organization and the increase of publishing costs.

DR. HARVEY E. THORPE: Mr. Chairman, I move the adoption of these recommendations.

DR. BEDELL: I second the motion.

CHAIRMAN ALLEN: Any further discussion? All in favor of increasing the dues from \$10 to \$15 for Active members and from \$3 to \$5 for Educational members, say "aye"; opposed, "no." The motion is carried unanimously.

CHAIRMAN ALLEN: The next order of business is the report of the Nominating Committee.

The Nominating Committee presents the name

of Dr. Bernard Becker for Trustee; Dr. Monte G. Holland for Secretary; Dr. A. Ray Irvine, Jr. for Treasurer, and Dr. Robert Moses for Assistant Secretary.

What is your will in regard to this report?

DR. BEDELL: I move the report be accepted.

(The motion was severally seconded, was put to a vote, and was carried unanimously.)

CHAIRMAN ALLEN: Dr. Becker has been nominated for Trustee. Are there other nominations from the floor? If not, a motion to close nominations and elect Dr. Becker is in order.

VOICE: I so move.

(The motion was duly seconded, put to a vote, and carried unanimously.)

CHAIRMAN ALLEN: Dr. Becker is elected the next Trustee.

Dr. Monte G. Holland has been nominated for Secretary. Are there other nominations from the floor? If not, a motion to close nominations and elect Dr. Holland is in order.

VOICE: Mr. President, I move that nominations be closed and that he be elected.

(The motion was duly seconded, put to a vote, and carried unanimously.)

CHAIRMAN ALLEN: The next officer to be elected is the Treasurer. Dr. A. Ray Irvine, Jr. has been nominated. Are there other nominations from the floor? If not, I shall entertain a motion to close nominations and elect Dr. Irvine.

DR. BEDELL: I so move.

(The motion was duly seconded, put to a vote, and carried unanimously.)

CHAIRMAN ALLEN: The next office is that of Assistant Secretary. Dr. Robert Moses has been nominated. Are there any other nominations from the floor? If not, a motion to close nominations and elect Dr. Moses is in order.

VOICE: I so move.

(The motion was duly seconded, put to a vote, and carried unanimously.)

CHAIRMAN ALLEN: Dr. Moses is elected.

The Board of Trustees wishes to recommend the election of three Honorary members at this time. They are Dr. Everett Goar, Dr. Francis Heed Adler, and Miss Mildred Weisenfeld. They are nominated by the Trustees. May we have a motion to approve and elect these nominees.

DR. BEDELL: I so move.

(The motion was duly seconded.)

CHAIRMAN ALLEN: It is duly moved and seconded that Dr. Goar, Dr. Adler and Miss Weisenfeld be elected Honorary members of the Association for Research in Ophthalmology.

(The motion was put to a vote and carried unanimously.)

CHAIRMAN ALLEN: At this time the meeting is open for any new business which may be brought to our attention. If there is no new business, a motion to adjourn the business session is in order.

VOICE: I so move.

(The motion was duly seconded, put to a vote, and carried unanimously.)

(The meeting adjourned sine die at 11:55 a.m.)

CONSTITUTION

ARTICLE I

NAME

The association shall be known as the Association for Research in Ophthalmology, Inc.

ARTICLE II

OBJECTS

The objects of the association are set forth in its application for a charter as follows: To encourage, promote, foster, and assist investigations and research in ophthalmology; in furtherance of the purpose of the corporation to purchase, lease, or otherwise acquire, hold, sell, lease, convey or otherwise dispose of real and personal property or any interest therein; to receive, hold, and invest funds and endowments and to receive and expand the income thereof, and to hold and dispose of such sums of money as may be deemed expedient; and generally to do any and all things which may be necessary or proper in connection with the objects and purposes of the corporation and which may not be contrary to law.

ARTICLE III

MEMBERS

Members of recognized ophthalmologic societies in the United States and Canada and other individuals especially qualified shall be eligible to membership when proposed in writing by a member of the Association and shall become members upon election by the Board of Trustees after the recommendation of the committee on admissions, upon payment of the dues provided for in the By-laws.

1. *Educational Membership.* Individuals may be elected to this class of membership during a period of graduate education in ophthalmology or related scientific fields and/or during a period of full-time research. In no instance may an educational membership be held longer than three years without reapplication.

2. *Active Membership.* Individuals not eligible for educational membership shall be elected to active membership but may choose either this class or sustaining membership.

3. *Sustaining Membership.* Individuals elected to membership in any class may voluntarily choose to become sustaining members.

4. *Life Membership.* Upon recommendation of the Board of Trustees and by a majority vote of the members of the Association present at its annual meeting, a member may be elected to life membership.

ARTICLE IV

BOARD OF TRUSTEES

There shall be a Board of Trustees consisting of six members, who shall be elected for a term of six years, and the secretary, the assistant secretary and the treasurer. The secretary, the assistant secretary and the treasurer of the Association will be members of the Board of Trustees ex officio. The senior member of the Board of Trustees shall be chairman and in the absence of the chairman, the member next in seniority shall act as chairman. At the first meeting of the board, the members shall draw lots for the purpose of determining which of them shall serve for one year, which for two, which for three, which for four, which for five, and which for six years; and the term of each member shall come to an end according to the lot which he shall have drawn. At the first annual meeting thereafter, a member of the Association shall be nominated for the Board of Trustees by the nominating committee of the Association for a term of six years in the place of the member whose term then expires, and thereafter at each meeting a member of the Board of Trustees shall be elected for a term of six years.

ARTICLE V

OFFICERS

The officers of the association shall be the chairman, other members of the Board of Trustees, the secretary, the assistant secretary and the treasurer.

ARTICLE VI

SECTIONS

Regional, national, or international sections of the association may be established by petition of twenty members in various geographical units either within or outside of the United States.

The officers of the sections shall be a section secretary appointed by the Board of Trustees and in addition such other officers as are elected by the section.

ARTICLE VII

NOMINATING COMMITTEE

The chairman of the Board with the approval of the Trustees shall appoint at the annual business meeting a nominating committee of three to serve for the following year. It shall be the duty of the nominating committee to nominate members for the office of secretary, assistant secretary, and treasurer and one member of the Board of Trustees.

ARTICLE VIII

DUTIES AND POWER OF OFFICERS AND TRUSTEES

The Board of Trustees shall have general charge of the affairs, funds and property of the association. It shall prepare the programs for the meetings of the association. It shall also appoint a committee on publication and a committee on admissions. It shall elect desirable applicants for membership approved by the committee on admissions. It shall have full power and it shall be its duty to carry out the purpose of the association according to the Charter, Constitution, and By-laws. A majority of its members shall constitute a quorum. Between meetings of the Board of Trustees, the executive power of the association shall be vested in the chairman of the Board of Trustees and the secretary and treasurer.

Chairman of the Board of Trustees. The chairman of the Board of Trustees is the chief executive of the association. He shall preside at all meetings, call all meetings, and perform all duties customary to the office.

Secretary. The secretary shall keep a record of the proceedings of all meetings; shall notify officers, trustees and members of committees of their election; certify official records; issue notices of meetings and perform all duties which may be required of him. He shall also obtain two copies of all scientific communications at the time the papers are read.

Assistant Secretary. The assistant secretary shall assist the secretary in all of his duties and shall accomplish such other duties as assigned by the chairman with the approval of the Board of Trustees.

Treasurer. The treasurer shall keep a list of all members. He shall have charge of all funds of the association; he shall keep the same and make disbursements therefrom as directed by the Board of Trustees. He shall furnish bond and have his accounts audited yearly by a certified accountant.

Section Secretary. Section secretaries shall act as assistants to the secretary and will serve as secretaries of sectional organizations.

The Section Chairman shall act as assistant to the chairman of the Board of Trustees in the execution of sectional meetings of the association. He shall preside at all meetings of the sections, call all such meetings and perform all executive duties customary to the office.

ARTICLE IX

ELECTION OF OFFICERS

A member of the association shall be elected to the Board of Trustees at each annual business meeting to serve for six years. The sixth year of his service as a member of the Board of Trustees will be served as chairman of the board and of the association.

The secretary, the assistant secretary and the treasurer of the association shall be elected by ballot at the annual business meeting to serve for one year.

Vacancies occurring in any office will be filled by the Board of Trustees for the unexpired term until the next annual election.

With the approval of the Board of Trustees, the secretary shall appoint a member of each section to act as section secretary for one year.

ARTICLE X

MEETINGS

The annual business meeting of the association shall be held at a time and place selected by the Board of Trustees. Additional scientific meetings may be held when due and proper notice is given by the secretary with the approval of the Board of Trustees.

Sectional meetings shall be held at a time and place selected by the section secretary and chairman and approved by the secretary and a member of the Board of Trustees.

ARTICLE XI

AMENDMENTS

Amendments to the Constitution may be made in the following manner: The amendment shall be written and shall be signed by three members of the association and submitted to the Board of Trustees at least thirty days before the annual meeting of the association. At the next annual meeting thereafter the Board of Trustees shall report at the meeting upon said proposed amendment. The amendment shall then be voted upon and two thirds of all the votes cast at the meeting shall be necessary for the adoption of the amendment.

BY-LAWS

ARTICLE I

MEETINGS

Meetings of the association shall be held at such time and place as the Board of Trustees shall determine. Twenty members shall constitute a quorum of the executive sessions of the meetings.

ARTICLE II

COMMITTEE ON ADMISSIONS

The Board of Trustees shall appoint annually a committee upon admissions consisting of three members. The names of all applicants shall be submitted to said committee which shall report its recommendations to the Board of Trustees.

ARTICLE III

DUES

The dues of the association shall be fifteen dollars per annum for active members, twenty-five dollars per annum for sustaining members and five dollars per annum for educational members, the fiscal year starting January first.

Dues shall be remitted for life members and honorary members.

To defray expense of sectional meetings, the section secretary may levy dues not to exceed one dollar per annum.

The treasurer, at the direction of the Board of Trustees, may distribute funds to meet unusual expenses of sections. This distribution of funds generally shall be in proportion to the size of the sectional membership.

ARTICLE IV

AMENDMENTS

The By-Laws may be amended in the same manner and with the same procedure as outlined for an amendment to the Constitution.

ARTICLE V

MEETINGS

All executive meetings shall be conducted according to Robert's Rules of Order unless specified otherwise in the Constitution and By-Laws.

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Hutchinson, Ben B.
McClure, G. David
Raiford, Morgan B.
Sawyer, Thomas R.
Shipley, Thorne
Struve, C. A.

GEOGRAPHICAL LIST

UNITED STATES

- ALABAMA**
- ANNISTON**
Gibbons, George W.
- BIRMINGHAM**
Burnham, Charles J.
Callahan, Alston
Dixon, Joseph M.
Grant, William D.
Kirkland, Theo Neal, Jr.
Miles, N. E.
Paul, Thomas O.
- FLORENCE**
Roberts, Shaler J., Jr.
- MONTGOMERY**
Benkwith, Karl B.
- ARIZONA**
- PHOENIX**
Carriker, Frederick R.
Lorenzen, Robert F.
McFarland, Paul E.
Zinn, Sheldon
- ARKANSAS**
- LITTLE ROCK**
Alford, T. Dale
Cosgrove, K. S.
Grayson, Merrill
- CALIFORNIA**
- ALHAMBRA**
Schiff, Frank S.
- BAKERSFIELD**
Baisinger, L. F.
Hart, Clinton E.
Simon, Glenn
Weyand, George M.
- BERKELEY**
Dickson, Owen C.
Flom, Merton C.
Gunderson, Ernest O.
Marg, Elwin
Sorenson, Lionel W.
Stoddard, Kenneth B.
Westheimer, Gerald
Yanof, Howard M.
- BEVERLY HILLS**
Faier, Herman I.
Gunzburg, Julian
Hare, Robert
Irvine, A. Ray, Jr.
Irvine, Rodman
Lordan, John P.
Ravdin, Emile M.
Somberg, Joseph
Stone, Howard H.
Weymouth, Frank W.
- BURLINGAME**
Schwartz, Ariah
- CHULA VISTA**
Mannis, Aaron A.
- EUREKA**
Iverson, Herman A.
Smith, Joseph G.
- FRESNO**
Donald, Russell A.
Rubin, Milton R.
Steinberg, Theodore
Trowbridge, Dwight H., Jr.
Whitten, Richard H.
- FULLERTON**
Bloomenthal, Sanford R.
- HAYWARD**
Levine, Julius
Raphael, Sylvan
- HUNTINGTON PARK**
Cury, Dahar
Gorrilla, L. Vincent
- INGLEWOOD**
Baker, Philip H.
Harwood, Samuel C.
Tandy, William
- LAFAYETTE**
Levin, Paul
- LA JOLLA**
Hultgen, William J.
Monsees, Wayne E.
- LANCASTER**
Hull, Forrest E.
- LONG BEACH**
Hertzog, Francis C., Jr.
Thornburgh, Robert G.
Wagner, William F.
- LOS ALTOS**
Nelson, Robert C.
- LOS ANGELES**
Abraham, Samuel V.
Apt, Leonard
Bach-y-Rita, Paul
Bartlett, Robert E.
Boyd, William H.
Carbajal, Ulysses
Christensen, Robert E.
Crescitelli, Frederick
Dayton, Glenn O., Jr.
Eby, Thomas M.
Ellis, Orwyn H.
Frisch, Michael R.
Hyde, Jane E.
Irvine, Wendell, C.
Kambara, George K.
Landegger, George P.
Mozersky, Samuel M.
Nugent, Maurice W.
Pettit, William A.
Planter, John
Popovich, Stephen J.
Robbins, Alfred R.
Roberts, Walter L.
Rome, Sol
Schaeffer, Alexander J.
Straatsma, Bradley R.
- LOS GATOS**
Banoff, Harry
Sitney, J. Jay
- MENLO PARK**
Stevenson, Thomas C.
- MONTEREY**
True, William R.
- NORTH HOLLYWOOD**
Fields, Jack
Nielsen, Ray H.
- OAKLAND**
Bell, Dudley P.
Caygill, Wayne M.
Garron, Levon K.
Gump, Millard E.
Howell, Homer P.
Schnoor, Thomas G.
- OJAI**
Hirsch, Monroe J.
- OROVILLE**
Bethel, R. D.
- PALM SPRINGS**
Grow, Kenneth A.
- PALO ALTO**
Bock, Rudolf H.
Flocks, Milton
Poore, John C.
Tanner, Owen R.
Winter, Frank C.
Zweng, Harold C.
- PARADENA**
Balding, Laurence G.
Johnson, Henrietta M.
Morgan, George E.
Riffenburgh, Ralph S.
- POMONA**
Donin, Jerry F.
Ryerson, F. Stuart
- REDONDO BEACH**
Gorchenko, Victor A.
- RESEDA**
Zankan, Leo
- RICHMOND**
Morgan, Meredith W., Jr.
- RIVERSIDE**
Biegel, Albert C.
Crawford, Walter J.
Mullen, Raymond S.
- SACRAMENTO**
Demorest, Myron H.
Kahler, Arthur R.
Reese, G. A.
Wagner, Alfred

MEMBERS

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SALINAS

Kraft, Frederick W.

SAN DIEGO

Bloomenthal, John
Merrill, H. Ross
Quint, J. Harley, Jr.

SAN FRANCISCO

Atkinson, Marshall B.
Barkan, Hans
Bettman, Jerome W.
Borley, W. E.
Campion, George S.
Colyear, Bayard H., Jr.
Cordes, Frederick C.
Crawford, Joseph W.
Dellaporta, Angelos
Eissler, Rolf
Erickson, Olive F.
Feeney, Lynette
Ferguson, William J., Jr.
Fine, Max
Foerster, Helenor C.
Friedman M. Wallace
Giles, Kenneth M.
Goodwin, Rufus C.
Hall, Thomas G.
Hanna, Lavelle
Harrington, David O.
Henry, Margaret
Hogan, Michael J.
Hosford, George N.
Jampolsky, Arthur J.
Kelley, Robert R.
Kimura, Samuel J.
Lachman, George S.
Loeb, Donald R.
McEwen, William K.
Mesirov, M. E.
Pischel, Dohrmann K.
Rodin, Frank H.
Rosenberg, Alan J.
Shaffer, Robert N.
Shekter, William B.
Steiner, Albert A.
Stevenson, Roger
Suran, Anita A.
Swett, Wilber F.
Tamler, Edward
Tour, Robert L.

SAN JOSE

Cook, Robert D.
Thygeson, Phillips
Vaughan, Daniel G., Jr.

SAN LEANDRO

Smith, Jaroud B., Jr.
Winn, W. E. Ted, Jr.

SAN MATEO

Abernethy, Rodney E.
Westsmith, Richard A.

SAN PEDRO

Zugsmith, George S.

SAN RAFAEL

Denicke, Ernest W.
McBain, Earle H.
Smith, Taylor

SANTA ANA

Anderson, Thomas W.
Forrest, Robert L.

SANTA BARBARA

Loutfallah, Michel

SANTA CRUZ

Trolan, Howard

SANTA MONICA

Bierman, Edward O.
Shapley, Albert
Sinskey, Robert M.
Smithson, Robert A., Jr.

SANTA ROSA

Aiken, Samuel D.
Lightfoot, Vernon F.

SHERMAN OAKS

Goodman, Sanders A.
Roberts, James E.

STOCKTON

Powell, James R.

TORRANCE

Nursall, John F.

VALLEJO

Madeley, H. Randall

VENTURA

Barnes, Charles R.

COLORADO

COLORADO SPRINGS

Haney, Lawrence O.
Wetzig, Paul C.

DENVER

Danielson, Ralph W.
Ellis, Philip P.
Long, John C.
Moorman, Lemuel T.
Rider, Mitchell B.
Swets, Edward J.
Tyner, George S.
Van Bergen, Thomas M.

GRAND JUNCTION

Rigg, James P.

KERSEY

Droegemueller, William H.

U. S. AIR FORCE ACADEMY

Fixott, Richard S.

CONNECTICUT

BRIDGEPORT

Frenkel, Henry H.

DARIEN

Ralph, Fenn T.
Williams, Frederick D.

FAIRFIELD

Lovekin, Louise G.

GREENWICH

Finlay, John R.
Tinkess, Donald E.

HARTFORD

Harris, Louis D.
Katz, Dewey
Mancall, Irwin T.
Unsworth, Arthur C.

HAMDEN

Wong, Andrew S.

NEW HAVEN

Blake, Eugene M.
Coulombre, Alfred J.
Fasanella R. M.
Stone, Leon S.
Zuckerman, Bernard D.

NORWICH

La Pierre, Warren W.

DISTRICT OF COLUMBIA

WASHINGTON

Alexander, Charles M.
Alper, Melvin G.
Day, Robert
Dubroff, Seymour
Fine, Ben S.
Holmes, Dorothy B.
Kimura, Kazuo
McTigue, John W.
O'Rourke, James F.
Rones, Benjamin
Silverstein, Arthur M.
Somers, Kenneth
Tousimis, Anastasios J.
Zimmerman, Lorenz E.

FLORIDA

CORAL GABLES

Horwich, Harry

FORT LAUDERDALE

Wold, Keith C.

GAINESVILLE

Casey, Ernest R.
Pinkson, Charles

JACKSONVILLE

Edwards, Thomas S.
Knauer, William J., Jr.

JAX

Lieurance, Richard E.

LAKELAND

Hester, Marion W.
Kummer, William M.

MIAMI

Jaffe, Norman S.
Levine, Oscar
McMackin, John V.
Norton, Edward W. D.

OCALA

Anderson, W. H., Jr.

PALM BEACH

Constantine, K. W.

PENSACOLA

Imas, Henry A.
Smith, Vernon L.

ST. PETERSBURG

Byrnes, Victor A.
Cope, Paul T.

SARASOTA

Dickinson, Thomas G.

TAMPA

Parsons, Hugh E.
Taylor, Joseph W., Jr.

WEST PALM BEACH

Preefer, Raymond R.

GEORGIA

ATLANTA

Baird, Mason J.
Calhoun, Phinizy F.
Calhoun, Phinizy F., Jr.
Crawford, H. C.
Hallum, Alton V.
Howell, Stacy C.
Smith, William A., Jr.
Stokes, Jack J.
Tabb, Granville W.

AUGUSTA

Edmondson, H. T.
Fair, John R.

COLUMBUS

Yoe, Lionel M.

EMORY UNIVERSITY

Brecher, Gerhard A.

MOULTRIE

Fokes, Robert E., Jr.

SAVANNAH

Buckhaults, W. W.

WAYCROSS

Clark, William S., Jr.

HAWAII

HONOLULU

Holmes, William John
Pang, Herbert G.
Pinkerton, Ogden D.

KAHULUI, MAUI

Kushi, Harold S.

IDAHO

POCATELLO

Clothier, William L.

TWIN FALLS

Cutler, Morton

ILLINOIS

BLOOMINGTON

Crowley, Frederick A.
Hartenbower, G. E.
Walsh, Rita

BLUE ISLAND

Field, Homer B.

CHAMPAIGN

Albers, Edward C.
Kresca, Frank J.

CHICAGO

Alfano, Joseph E.
Banerjee, Renuka
Belkows, John G.
Brown, E. V. L.
Clark, James W.
Cowen, Jack F.
Cushman, Beulah
de Francois, Walter
Guibor, George
Haas, Joseph S.
Henry, Marvin D.

Hoeltgen, Maurice M.
Hughes, William F., Jr.
Iser, Gilbert
Klien, Bertha A.
Krimmer, Burton M.
Gronfeld, Peter C.
Lieberman, Howard L.
Mann, William A.
Matusak, Lucian R.
Merz, Earl H.
Meyer, Samuel J.
Nethercut, Glenway
Newell, Frank W.
Pearlman, Maurice D.
Potts, Albert M.
Puntenny, Irving
Pushkin, Edward A.
Riesen, Austin H.
Roper, Kenneth L.
Rosenberg, William
Schall, Samuel M.
Scheffler, Milton M.
Schultz, Abraham
Shapira, Theodore M.
Shoch, David Eugene
Skowron, John J.
Snydacker, Daniel
Sternberg, Paul
Stillerman, Manuel L.
Stonehill, Alfred A.
Swiontkowski, Stanley D.
Tepper, Norman N.
Vail, Derrick
Van Wien, Stefan
Vickery, Robert David
Wescott, Virgil
Zekman, Theodore N.

CICERO

Lhotka, F. M.

DES PLAINES

Kreft, Warren W.

ELMHURST

Black, Chester J.

EVANSTON

Gerber, Margaret
Lawson, Lawrence J., Jr.
Soper, Gail R.

GLENCOE

Sarnat, Leonard A.

GLENVIEW

Patience, Hansi R.

LA SALLE

Gallardo, Edward

MACOMB

Weston, Charles L.

OAK PARK

Fitzgerald, James R.
Good, Palmer
Kirk, Harold C.
McDonald, James E.
Smith, Warren F.
Theobald, G. D.

PEORIA

Wyman, George J.

QUINCY

McReynolds, William U.

ROCKFORD

Pritikin, Roland I.

SROKIE

Hurwitz, Paul
Monninger, Robert H.

SPRINGFIELD

Wiesbaum, Byron M.

WILMETTE

Brown, David V.

INDIANA

BLOOMINGTON

Allen, Merrill J.
Heath, Gordon G.
Hofstetter, William

EVANSVILLE

Wesson, Thomas W.

FORT WAYNE

Rothberg, Maurice
Young, Robert L.

HAMMOND

Kuhn, Hedwig St.

INDIANAPOLIS

Cuthbert, Marvin
Dyar, Edwin W.
Harger, Robert W.
Mann, Mortimer
Masters, Robert J.
Rutherford, Cyrus W.
Schlaegel, Theodore F., Jr.
Taube, Jack I.
Wagoner, Robert A.
Wilson, Fred M.

LAFAYETTE

Van Buskirk, Edmund L.

LA PORTE

Philbrook, S. S.

MUNCIE

Morris, Jean W.

NEW CASTLE

Burnett, Arthur B.

RICHMOND

Allen, Robert T.

SOUTH BEND

Cassady, J. Vernal
Cassady, John R.

WHITING

Apter, Julia T.

IOWA

BURLINGTON

Walker, Glenn L.

CARROLL

Sullivan, John V.

CEDAR RAPIDS

Noe, Carl A.
Sawyer, Thomas R.

CLINTON

Weib, Elmer P.

MEMBERS

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DES MOINES

Downing, Arthur H.
Lambrecht, Paul

FORT DODGE

Kluever, H. C.

IOWA CITY

Alexander, Rose C.
Allen, Lee
Arnott, George P.
Blodi, Frederick C.
Boeder, Paul
Braley, Alson E.
Burian, Hermann M.
Ferguson, Edward C., III
Janes, Ralph G.
Leinfelder, P. J.
Richards, Richard D.
Rubin, Melvin L.
Schultz, Richard O.
Van Allen, Maurice W.
Von Noorden, Gunter K.
Watzke, Robert C.
Wise, Arthur C.

MARSHALLTOWN

Wolfe, Otis D.
Wolfe, Russell M.

OSKALOOSA

Atkinson, George S.

SIOUX CITY

Reeder, James E., Jr.

WATERLOO

Phelps, Gardner D.

KANSAS

ATCHISON

Bribach, E. J.

LAWRENCE

Hall, James L.

KENTUCKY

LOUISVILLE

Heitger, Joseph D.
Keeney, Arthur H.
MacDonald, Roderick, Jr.
Moran, Charles T.
Pfingst, Harry A.
Rabb, Maurice F., Jr.
Townes, C. Dwight

LOUISIANA

ALEXANDER

Simmonds, Noel T.

GREYNA

Adair, Bonnie L.

LAFAYETTE

Sonnier, William, Jr.

METAIRIE

Holland, Monte G.

NEW ORLEANS

Adler, Hartwig
Allen, James H.
Bahn, Charles A.
Bahn, Gustav C.
Barber, Aelita N.
Boles, William M.

Chulavachana, Tavisak
Clark, William B.
Dabezies, Oliver H., Jr.
Ellis, George S.
Fisher, Earl, Jr.
Haik, George N.
Jimenez, Timoteo
Leckert, Edmund L., Jr.
MacDonald, Roderick, Jr.
Rosenthal, J. William
Rumage, Joseph P.
Schoel, Robert E.

RUSTON

Harms, Harold H.

SHREVEPORT

Gray, Leon F.

MAINE

BANGOR

Osler, Jay K.

LEWISTON

Tchao, Jou S.

PORTLAND

Maier, Paul

WATERVILLE

Dennis, Richard H.
Hill, Howard F.

MARYLAND

BALTIMORE

Abrahams, Irwin W.
Brumback, Joseph E., Jr.
Duke, James R.
Goldberg, Herman K.
Hoover, Richard E.
Langham, Maurice E.
Lawrence, Carteret
MacLean, Angus L.
Maumenee, Alfred E.
Naquin, Howard
Patz, Arnall
Rowland, Louise S.
Wolff, Stewart M.
Wood, Ronald M.
Woods, Alan C.

BETHESDA

Chalfant, W. Paxson, Jr.
Gunkel, Ralph D.
Hart, William H.
Livingston, Robert B.
Macri, Frank J.
Peckham, Robert H.
Schacter, Bernard
van Sallmann, Ludwig

CHEVY CHASE

Glew, William B.

MASSACHUSETTS

BOSTON

Allen, Henry F.
Andrews, John S., Jr.
Balazs, Endre A.
Beetham, William P.
Boruchoff, S. Arthur
Cabbit, Henry L.
Casten, Virgil C.
Chandler, Paul A.
Chisholm, Julian F., Jr.
Cogan, David G.
Donahue, Hugh C.

Dunphy, Edwin B.
Futtermann, Sidney
Grant, W. Morton
Gundersen, Trygve
Heath, Parker
Howe, Arthur F.
Jakus, Marie A.
Kaufman, Herbert E.
Kern, Harold L.
Kinoshita, Jin H.
Kupfer, Carl
Kuwabara, Toichiro
Lichman, Sumner D.
Lo-Presti, Joseph J.
Martin, S. Forrest
Merola, Lorenzo O.
Murray, Edward S.
Regan, Charles D. J.
Schepens, Charles L.
Sloane, Albert E.
Snyder, John C.
Sweebe, Edward C.
Verhoeff, F. H.
Ziv, Benjamin
Zucker, B. B.

BROOKLINE

Cherr, Donald
Stone, William

CAMBRIDGE

Wald, George

LOWELL

Leahey, Brendan D.

MEDFORD

Carpenter, Russell L.

NORWOOD

Ryan, William F.

SPRINGFIELD

Bick, Malcolm W.
Corcoran, George B., Jr.
Perlman, Arnold R.
Waugh, Richey L., Jr.

WORCESTER

Broggi, Richard J.
Holzer, William F.
Myers, Roscoe W.
Whitney, Percy T.
Yasona, Elton

MICHIGAN

ANN ARBOR

Alpern, Mathew
Falls, Harold F.
Frailick, F. Bruce
Henderson, John W.
Scharenberg, Konstantin
Sippel, Theodore O.
Smillie, John W.
Wolter, J. Reimer

BAY CITY

Heuser, Harold H.

DEARBORN

Bialik, Michael H.
Markey, Frank R.
Sallee, William T.

DETROIT

Burroughs, Roswell G.
Crossen, Robert J.
Davies, Windsor S.

- Dunlap, Henry A.
Frey, James L.
Guyton, Jack S.
Johnson, David S.
Kinsey, V. Everett
Kock, John F., Jr.
Lepard, C. W.
Lerner, Leonard H.
Neeb, Walter
Olson, James A.
O'Rahilly, Ronan
Pugh, Howard C.
Reddy, D. Venkat
Reid, Wesley G.
Ruedemann, A. D.
Ruedemann, A. D., Jr.
Scarney, Herman D.
Schane, David
Schimek, Robert A.
Sugar, H. Saul
- FLINT
Barbour, Fleming A.
Rundles, Walter Z.
- GROSSE POINTE WOODS
Marshall, James R., Jr.
- JACKSON
McGarvey, William E.
Wholihan, John W.
- LINCOLN PARK
Flaherty, Norman W.
Wehr, Maurice B.
- NILES
Moore, T. Scott
- TRAVERSE CITY
Beall, John G.
Weihs, Jack E.
- WYANDOTTE
Barsky, David
- YPSILANTI
Petrohelos, Manousos A.
- MINNESOTA
DULUTH
Fellows, M. Fording
Hilding, Anderson C.
- LITTLE FALLS
Johnson, Douglas L.
- MINNEAPOLIS
Fink, Walter H.
Fowlks, W. L.
Friedman, Harry S.
Gruber, Louise
Halberg, Franz
Hammerstad, Lynn M.
Harris, John E.
Hoffman, Walter L.
Jerome, Bourne
McCannel, Malcolm A.
Sandt, Karl E.
Schwartz, Virgil J.
Shapiro, Irving
Shaw, Howard A.
Simonson, Ernst
Walsh, Francis M.
Wendland, John P.
- ROCHESTER
Bair, Hugo L.
Benedict, William L.
- Henderson, John W.
Holenhorst, Robert W.
Martens, Theodore G.
Ogle, Kenneth N.
Rucker, C. Wilbur
Sabin, Frederick C.
Sheard, Charles
Steinmetz, Rodney D.
- ST. CLOUD
Wenner, W. T.
- ST. PAUL
Burch, Edward P.
Grant, Hendrie W.
Monahan, Robert H.
O'Kane, Thomas W.
Strate, Gordon E.
- MISSISSIPPI
GREENVILLE
Gamble, Lyne S.
- GULFPORT
Flagg, G. B.
- JACKSON
Johnson, Samuel B.
Mayer, Leo L.
- MISSOURI
CAPE GIRARDEAU
Wolff, Paul G.
- CLAYTON
Bryan, James H.
Drews, Leslie C.
Freeman, David M.
Hildreth, H. Rommel
Israel, Milton R.
- COLUMBIA
Buesseler, John A.
- JEFFERSON CITY
Stauffer, Harry B.
- KANSAS CITY
Calkins, Larry L.
Curran, Desmond
Eubank, William R.
Lemoine, Albert N., Jr.
McKee, Joseph W.
McLeod, John
May, James W.
Padfield, Earl G., Jr.
Robison, James T., Jr.
Rufe, John R.
Shaad, Dorothy J.
- MEXICO
Rouise, David M.
- ST. LOUIS
Alvis, Edmund B.
Becker, Bernard
Bisno, Daniel
Boldizar, A. G.
Cibis, Paul A.
Constant, Marguerite A.
Drews, Robert C.
Enoch, Jay M.
Hartstein, Jack
James, William M.
Ley, Albert P.
Luedde, Philip S.
Mattis, Robert D.
- Miles, Paul W.
Miller, James E.
Moses, Robert A.
Post, Martin H., Jr.
Rosenbaum, Harry D.
Sanders, T. E.
Schwartz, Frederick O.
Shah, Anwar
Shahan, Philip T.
Venable, Howard P.
Yamashita, Tsuyoshi
- MONTANA
BILLINGS
Morledge, Charles C.
- NEBRASKA
LINCOLN
Paulson, Hubert O.
Thomas, Richard L.
- OMAHA
Alliband, George T.
Filkins, John C.
Gifford, Harold
Judd, John H.
McIntire, Walden C.
Morrison, W. Howard
Rasgorshek, Robert H.
Swab, Charles M.
Truhlsen, Stanley M.
- NEVADA
RENO
Magee, George R.
- NEW HAMPSHIRE
BERLIN
Ingalls, Raymond G.
- HANOVER
Auten, Hanford L.
Sullivan, Paul B.
- NEW JERSEY
ASBURY PARK
Fisher, James A.
- ATLANTIC CITY
Harley, Robison D.
- BOUND BROOK
Levy, Abram
- CAMDEN
Barnshaw, Harold D.
Meyer, George P.
- DEAL
Amdur, Joseph
- EAST ORANGE
Jaekle, Charles E.
- FAIRLAWN
Seligson, Alvin
- FAR ROCKAWAY
Goldsmith, Maximilian O.
- HACKENSACK
Berke, Raynold N.
- HADDONFIELD
Finley, John K.

JERSEY CITY

Cinotti, Alfonse A.

LONG BRANCH

Roberts, N. Craig

NEWARK

Adelman, Benjamin B.

OCEAN CITY

Pettit, Paul H.

PASSIAC

D'Amico, Thomas V.
Ehrenfeld, Edward
Lang, Richard E.
Silverstein, Arthur L.

PLAINFIELD

Samuels, S. Lawrence

PRINCETON

Abrams, Henry
Lascherer, E. Frederick

SHORT HILLS

Fonda, G. E.

SUMMIT

McAlpine, Paul T.

TRENTON

Baisis, Bernard A.
Murto, Robert E.
Sacks-Wilner, Erwin
Sharbaugh, George B.
Wilner, Arthur S.

NEW MEXICO

ALBUQUERQUE

Dillahun, Jack A.
Schonberg, Albert L.

NEW YORK

ALBANY

Bedell, Arthur J.
Ward, Robert H.

AMSTERDAM

Fethke, Norbert
Malec, Henry W.

ASTORIA

Weingeist, Samson

AUBURN

Platt, Edward S.

BAY SHORE

Bussey, Frank R.
Solomon, Yale

BAYSIDE

Goodstein, Seymour

BINGHAMTON

Landers, Philip H.
Tobin, Henry L.
Walling, Henry G.
Werner, George B.

BRONX

Burman, Daniel
Fleischner, Alois L.

BROOKLYN

Ajello, Dominick A.
Beery, Edwin N.
Bodian, Martin
Bonadia, Calogero
Brackup, Alvin H.
Cholst, Mortimer
Evans, John N.
Fenton, Robert H.
Fink, Austin I.
Fradin, Seymour
Goldberg, Robert T.
Jampel, Robert S.
Landesberg, Jacques
Levine, George
Levitt, Jesse M.
Mandelbaum, Joseph
Marinakos, Charles S. G.
Marton, Herbert
Najac, Harold W.
Rosenberg, Abner S.
Rosenthal, Benjamin C.
Sands, Abraham M.
Schwartz, Bernard
Sherman, Maurice E.
Stahl, Norman O.

BUFFALO

Addington, Charles H.
Bennett, Arthur L.
Brennan, James W.
Cowper, Alexander R.
Fial, Edward A.
Fowler, James G.
Freeman, Sheldon B.
Higgs, Howard H.
Howard, William M.
Jones, W. Yerby
LeWin, Thurber
Luhr, John P.
Naples, Ange S.
Noell, Werner K.
Olmsted, K. Elizabeth P.
Reitz, Herbert R.
Schopp, Robert C.
Smallen, Benjamin

CORNING

Hoffman, Parker M.

ELMHURST

Flam, H. Leonard
Sanchez, Erlinda T.

ELMIRA

Boland, William T.
Voorhees, Charles H.

FAIR ROCKAWAY

Greenberg, Milton

FLUSHING

Katlan, Nathaniel R.
Kellerman, Leo D.
Sass, William O.

FOREST HILLS

Charap, Bertram W.
Cohen, Abraham

GARDEN CITY

Griffith, Perry P.
Kieser, Carl E.

GLEN COVE

Boyd, James L.

GLEN FALLS

Mintz, Maxwell A.
Ryan, Edward P.

GREAT NECK

Branower, Gerald
Kaufman, Ira H.
Masor, Philip L.

HEMPSTEAD

Bruno, Mary G.

ITHACA

Pritchard, Dale B.

JACKSON HEIGHTS

Echols, Porter B., Jr.
Presto, Ernest C.
Weinstein, Albert

JAMAICA

Douglas, Edward M.
Goldberg, Harry

JAMESTOWN

Franks, Myron B.

KEW GARDENS

Rubin, Herbert S.

LANCASTER

Palmer, Milton A.

LAWRENCE

Wiener, Howard E.

LINDENHURST

Mason, David J.

LOCKPORT

Morgana, Dante J.

LONG ISLAND CITY

Grimaldi, Dominick

MASSAQUA

Bergmann, Robert B.

NEW HYDE PARK

Chatzino, Albert B.

NEW YORK

Bassen, Edward J.
Berens, Conrad
Berliner, Milton L.
Billet, Edwin
Bloomfield, Sylvan
Bonaccolto, Girolamo
Born, John H.
Breakey, Arnold S.
Breinin, Goodwin M.
Bruce, Gordon M.
Byron, Herve M.
Campbell, Charles J.
Campbell, Francis P.
Carr, Ronald E.
Carter, George Z.
Carter, Vincent, Jr.
Castroviejo, Ramon
Chambers, A. L., II
Chamlin, Max
Chawchureh, Endliam
Chi, Helen H.
Chin, Newton B.
Clark, Graham
Cohen, Irwin J.
Cole, Helen G.
Cole, John G.
Coles, Robert S.
Constantine, F. H.
Consul, Bishan N.

Curtin, Brian J. H.
 Danforth, Edward P.
 Day, Robert M.
 Devi, Anima
 DeVoe, Arthur G.
 Dische, Zacharias
 Doctor, Daniel W.
 Dunlap, Edward A.
 Dunnington, John H.
 Eliasoph, Ira
 Ellsworth, Robert M.
 Epstein, Sidney S.
 Esterman, Benjamin
 Feinstein, Robert H.
 Feldstein, Morris
 Fogarty, Terence P.
 Fried, Joseph J.
 Friedman, Benjamin
 Gartner, Samuel
 Givner, Isadore
 Goldberg, Bernard
 Goodman, George
 Goodside, Victor
 Gordon, Dan M.
 Gorin, George
 Guy, Loren P.
 Halberg, G. Peter
 Hartshorne, Isaac
 Jacobson, Jerry H.
 Herman, John S.
 Jones, Ira S.
 Kahan, Edmund
 Kara, Gerald B.
 Katzin, Herbert M.
 Keil, Francis C., Jr.
 Kimmelman, David B.
 Knapp, Arnold
 Knapp, Arthur A.
 Knapp, Phillip
 Kochman, Richard S.
 Koppel, Zoltan I.
 Kornzweig, Abraham L.
 Krohn, David L.
 Kronenberg, Bernard
 Krug, Joseph H.
 Kumnick, Lillian S.
 Lasker, Mary
 Laval, Joseph
 Levene, Ralph Z.
 Links, Arthur
 Lisman, Jack V.
 Locatcher-Khorazo, Deborah
 Loewenfeld, Irene E.
 Longfellow, Don W.
 Lowenstein, Otto
 Lubkin, Virginia
 McDonald, George
 McLean, John M.
 Mamelok, Alfred E.
 Marcus, Arthur A.
 Merriam, George R., Jr.
 Nathaniel, Arthur
 O'Connor, George R.
 Paton, R. Townley
 Payne, Brittain F.
 Pearlstone, Arnold D.
 Perera, Charles A.
 Posner, Adolph
 Proctor, Malvin
 Regan, Ellen F.
 Reese, Algernon B.
 Rittler, M. Catherine
 Roberts, Bernard A.
 de Roeth, Andrew, Jr.
 Romaine, Hunter
 Sauer, John J.
 Schachar, Walter S.
 Schachne, Lewis
 Schlossman, Abraham
 Schneider, Julius
 Schultz, Sigmund

Shafer, Donald M.
 Silverman, Stanley M.
 Smelser, George K.
 Smith, Byron
 Snyder, Stuart S.
 Starr, Wilson C.
 Stasior, Orkan G.
 Sturman, Robert M.
 Sudarsky, R. David
 Tabowitz, David
 Teng, Chih Chiang
 Theodore, Frederick H.
 Troutman, Richard C.
 Turtz, Arnold I.
 Tusak, Ervin A.
 Vesey, Frank
 Wadsworth, Joseph A. C.
 Webster, David H.
 Weimar, Virginia L.
 Weintraub, Alfred
 Wexler, David
 Wheeler, Maynard C.
 Wise, George N.
 Young, Morris

NIAGARA FALLS

Goetzman, Arthur C.

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Sheldon, Maurice G.

PEARL RIVER

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Baum, Gilbert

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Cohen, Irving

ROCHESTER

Caccamise, William C.
 Gipner, John F.
 Knoll, Henry A.
 Lerman, Sidney
 Levy, Chauncey F., Jr.
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 Sabey, Peter K.
 Snell, Albert C., Jr.
 Sullivan, Charles T.

ROCKVILLE CENTRE

Preefer, Charles

ROME

Reid, Frederick K.

ROSLYN HEIGHTS

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 London, William

RYE

Simonton, John T.

SCHENECTADY

Polesny, Karel
 Sykowski, Peter S.

STATEN ISLAND

Howard, Royal M.
 Taj Meher, Amir H.

SYRACUSE

Gillette, David F.
 Kaiz, I. Herbert
 McGraw, James L.
 Marlow, Searle B.
 Sullivan, John J.

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Valone, Richard J.

VALLEY COTTAGE

Cumming, Edith L. Wentworth

WANTAGH

Esbin, Leo

WARSAW

Fountain, Newland W.
 Leachman, John W.

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Atkinson Walter S.

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 Morales, Gustavo

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Galvin, Miles A.

WOODSIDE

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ASHVILLE

Odum, Robert E.

CHARLOTTE

Ghent, Thomas D.

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 McPherson, Samuel D., Jr.
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GOLDSBORO

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WINSTON-SALEM

Holt, Lawrence B.
 Perreten, Frank A.
 Roberts, R. Winston
 Weaver, Richard G.

NORTH DAKOTA

GRAND FORKS

Prochaska, Leonard J.

MINOT

Kohl, Darwin L.

OHIO

AKRON

Krichbaum, Franklin M.
 Mathias, Daniel W.

ALLIANCE

King, George L.

CINCINNATI

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 Abrahamson, Ira A., Jr.

Asbury, Mark K.
Asbury, Taylor
Ascher, Charles K. W.
Brown, Albert L.
Freemond, Alan S.
Ginsberg, Joseph
Kemper, Robert A.
Lyle, Donald J.
Reid, Horace W.
Sakler, Barnet R.

CLEVELAND

Adams, Elliot O.
Adelstein, Nelson J.
Ballintine, Elmer J.
Bennett, James E.
Binder, Hertha F.
Binder Rudolf F.
Bortoff, Alexander
Brandwan, Samuel R.
Budd, Francis X.
Chamberlain, W. P., Jr.
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Fleming, David G.
Gans, Jerome A.
Gans, Morris E.
Guth, Sylvester K.
Hardesty, Hiram H.
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Hertner, John E.
Johnson, Lorand V.
Kazdan, Philip
Kennedy, Roscoe J.
Keyes, John E. L.
Kirk, Robert C.
Kohn, Howard D.
Kottler, Saul
Nicholl, Russell J.
Phillips, Josephine D.
Rosner, Robert S.
Schwarz, Gerald T.
Smith, Clarence G.
Sperelakis, Nick
Thomas, Charles I.
Volk, David
Volk, Myron
Waitzman, Morton B.
Weekley, Robert D.
Wolpaw, Benjamin J.

COLUMBUS

Barton, Stanley L.
Battles, Morris L.
Blackwell, H. Richard
Blackwell, O. Mortenson
Ellerbrock, Vincent J.
Fry, Glenn A.
Havener, William H.
Hebbard, Frederick W.
Knox, George W.
Lipetz, Leo E.
Magnuson, Robert H.
Makley, Torrence A.
Perry, Calude S.
Prince, Jack H.
Stine, George T.
Suie, Ted
Wachtl, Carl

DAYTON

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Thomas, Edward R.

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Van Dyke, Donald P.

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Ellenberger, Carl

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PARMA

Graham, John H.

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Steinberg, Bernhard
Taylor, Frank W.

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Stertzbach, C. W.

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Ozment, Thomas L.

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Christensen, Leonard
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Martz, George E.

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Carmichael, Paul L.

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Adler, Francis H.
Ashodian, Mila J.
Askovitz, Samuel I.
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Belmont, Owen
Brav, Solomon S.
Clough, Howard K.
Cohen, Louis B.
Cowan, Alfred
Ellis, Richard A.
Fajardo, Romeo V.
Frayner, William C.

- Gettes, Bernard C.
Goren, Seymour B.
Green, Harry
Gibson, Glen G.
Hallett, Joseph W.
Hedges, Thomas R., Jr.
Krewson, William E., III
Krishna, Narendra
Leopold, Irving H.
Lipsius, Edward I.
Lutman, Frank C.
McDonald, P. Robb
Mullen, Carroll R.
Naidoff, David
Reese, Warren S.
Sarin, Lov K.
Scheie, Harold G.
Sery, Theodore W.
Spaeth, Edmund B.
Spaeth, Philip G.
Trueman, Robert H.
Waldman, Joseph
Weaver, Harry S., Jr.
Wolkowicz, Michael I.
Zentmayer, William
- PITTSBURGH**
Berk, Myles M.
Billings, Robert J.
Bronk, Henry N.
Davies, Robert H.
Dunbar, John C.
Evans, Thomas M.
Everett, William G.
Geeseman, George R.
Goldberg, Sol
Hoffman, Franklin D.
Krieger, A. A.
Lehman, Robert N.
Linn, Jay G., Jr.
Linn, Jay G., Sr.
McCaslin, Murray F.
Novak, Joseph F.
Rohm, Robert F.
Saul, Robert W.
Statti, Louis W.
Steinberg, Abraam
Thorpe, Harvey E.
Veenis, C. Y.
Weisser, Charles W.
Wickerham, Earl P., Jr.
Wolken, Jerome J.
- READING**
Craig, Paul C.
Quereau, James V. D.
Souders, Benjamin F.
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Capriotti, Octavius A.
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Nadler, M. Princeton
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Riddle, Ransford J.
Snyder, M. Wilson
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PROVIDENCE
Ross, Milton G.
Stephens, H. Frederick
- SOUTH CAROLINA**
CHARLESTON
Croffhead, George S.
Vallotton, William W.
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Stokes, Julius H.
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Brown, Robert A.
Jervy, James W., Jr.
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ABERDEEN
Rudolph, Edward A.
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Kilpatrick, William R. J.
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Palmerton, E. S.
- YANKTON**
Willcockson, Thomas H.
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CHATTANOOGA
Arnold, I. Lee
- JOHNSON CITY**
Campbell, E. Malcolm
Wilson, John M.
- KINGSFORT**
Boles, James H.
- KNOXVILLE**
Benedict, Walter H.
Leach, Robert S.
- MEMPHIS**
Deutsch, Alice R.
Krause, Arlington C.
Lewis, Phillip M.
Rychener, Ralph O.
- NASHVILLE**
Bounds, George W., Jr.
Driver, L. Rowe
Lawrence, G. Allen
Patterson, John W.
Rowe, Fred A., Jr.
- TEXAS**
ABILENE
Scale, Hubert J.
- AMARILLO**
McKay, Edward D.
Murphy, Weldon O.
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Davidson, Morris
Hilgartner, H. L.
Key, Samuel N., Jr.
Leggett, Carey, Jr.
Thompson, Hardy, E., Jr.
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Caldwell, Pearson C.
- BROWNSVILLE**
McAlester, A. W., III
- CORPUS CHRISTI**
Gill, Earl K.
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Browning, Carroll W.
Burnside, Ronald M.
Byers, Jerome L.
Cary, Edward H.
Grafton, Edwin G.
Melton, Carlton E., Jr.
Quinn, Lester H.
Thomas, Maxwell
- EL PASO**
Schuster, Stephen A.
- FORT HOOD**
Chambers, Arthur L., II
- FORT WORTH**
Beasley, Harold
Conner, William C.
Fleming, T. C.
McKenzie, Walter H.
Merrill, David L.
Maxwell, Earl
- GALVESTON**
Gingrich, Wendell D.
Potter, William B.
Robertson, Gaynelle
- HOUSTON**
Blau, Robert I.
Daily, Louis, Jr.
Daily, Ray K.
Dukes, Charles D.
Feldman, Gerald L.
Fletcher, Mary C.
Ford, Donald P.
Girard, Louis J.
Goar, Everett L.
Griffey, Edward W.
Knolle, Guy E.
Lehigh, Richard, Jr.
Vanzant, Thomas J.
Wahlen, Henry E.
- SAN ANTONIO**
Matthews, John L.
Mims, James L., Jr.
Sykes, John H. J.
Weixel, Francis X.
- TEMPLE**
Veirs, Everett R.
- VICTORIA**
Bade, Craig P.
- UTAH**
PROVO
Oaks, Lewis W.
- SALT LAKE CITY**
Muir, Everett B.
Smith, Homer E.
- VERMONT**
BURLINGTON
Cunningham, John C.
Hanna, Colvin

VIRGINIA

ARLINGTON

Mitchell, Robert H.

CHARLOTTESVILLE

Brunish, Robert
Burton, Edwin W.
Spence, George

DANVILLE

Byerly, Baxter H.

LYNCHBURG

Berkley, William L.
Hobbs, Frank I.

QUANTICO

McArtor, James R.

RICHMOND

Geeraets, Walter J.
Guerry, duPont, III
Rein, Walter J.
Sheppard, L. Benjamin
Stratford, Thomas P.
Wiesinger, Herbert
Williams, Richard K.

ROANOKE

Stone, W. Conrad
Young, Charles A., Jr.

WINCHESTER

McGuire, William P.

WASHINGTON

BELLEVUE

Milam, Daniel F., Jr.

BELLINGHAM

Kaiser, Robert F.

BREMERTON

Benson, Clifton E.

LONGVIEW

Hill, Robert V.

PULLMAN

Young, Francis A.

BENTON

Maynard, Robert E.

SEATTLE

Hargias, James L.
Hungerford, Louis N.
Jensen, Carl D. F.
Lyda, Wood
Peter, Philip A.
Peterson, Walter C.
Shumate, Robert E. L.
Soll, David B.

SPOKANE

Gates, Charles L.
Richter, William C.
de Roeth, Andrew F. M.

TACOMA

Ward, James F.

WALLA WALLA

Hogenson, Clifford D.
Stevens, Ralph W.

WENATCHEE

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WEST VIRGINIA

CHARLESTON

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Polan, Charles M.

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WISCONSIN

APPLETON

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Mansheim, Bernard J.

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Engerman, Ronald L.

MILWAUKEE

Blankstein, Samuel S.
Bolger, James V.
Carl, E. Franklin
Gaynon, Irwin E.
Hitz, John B.
Holm, Arvid G.
Schalmo, E. H.

MONROE

Mings, Dwain E.

NEENAH

Kirchgeorg, Clemens

RACINE

Kadin, Maurice

FOREIGN COUNTRIES

PUERTO RICO

CAGUAS

Buonomo, Emigdio A.

SAN JUAN

Fernandez, Guillermo J.

SANTURCE

Buxeda, Roberto
Maestre, Federico J.
Margarida, Carlos J.

CANADA

ALBERTA

EDMONTON

Duggan, John W.
Marshall, M. R.

MANITOBA

WINNIPEG

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Quigley, John H.

ONTARIO

BELLEVILLE

Walcott, John M. L.

KINGSTON

Rosen, David A.

LONDON

Dyson, Charles

NIAGARA FALLS

Eager, R. F.

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Fraser, Wilbur F.

TORONTO

Basu, Prasanta K.
Bishop, Harding E.
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Levitt, Herbert
McCulloch, Clement
MacDonald, Roy K.
Ormsby, Hugh L.
Shusterman, Morris

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Boxer, Louis
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Cloutier, Roland
Langman, Jan
Locke, John C.
Viger, R. J.

BRITISH COLUMBIA

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Simpson, Derek G.

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Wiggins, Robert L.

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Bailey, N. L.

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Taniguchi, Yoshiaki

TOKYO

Hagiwara, Hogara

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Arribas, Neva P.
De Ocampo, Geminiano
Fernando, Antonio N.

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Quilala, Francisco V.

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ANTWERP

Neetens, Adolphe

GHENT

Francois, Jules

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Duke-Elder, Lady Phyllis
Duke-Elder, Sir Stewart

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GENOVA

Grignolo, Antonio

ROME

Strampelli, Benedetto

SPAIN

BARCELONA

Barraquer, Joaquin

MADRID

Carrasquillo, Honorio F.

SWITZERLAND

GENEVA

Blum, John

ZURICH

Huber, Alfred

CENTRAL AND LATIN AMERICA

ARGENTINA

CORDOBA

Urreta-Zavalin, Alberto, Jr.

BRAZIL

BAHIA

de Andrade, Cesario

MINAS GERAIS

Dasilva, Antonio I.

COSTA RICA

SAN JOSE

de la Cruz, Edgar

MEXICO

MEXICO, D. F.

Camacho, Enriqueta
Puig-Solanes, M.
Wudka, Enrique

PERU

LIMA

Barrere, Luciano E.
Raffo, Julio C.

VENEZUELA

CARACAS

Grom, Edward

WEST INDIES

CUBA

HAVANNA

Ferrer, Horatio

FUTURE MEETINGS

NATIONAL

NEW YORK CITY

JUNE, 1961

PROGRAM

MONDAY, JUNE 26

9:00 A.M.

1. Formation and Distribution of Chick Lens Proteins.
Jan Langam.
2. Effect of Ionizing Radiation on Protein and Carbohydrate Metabolism in the Rat Lens.
Sidney Lerman and Anima Devi.
3. Changes in Various Fractions of Ribonucleic Acid of Rabbit Lenses during Aging.
Zacharias Dische, Ginerva Zelmis and Nina Larys.
4. Chromosomal Study of Corneal Cells of Rabbit Grown in Tissue Culture.
P. Sarkar and P. K. Basu.
5. Resonant Frequency of a Rod E. R.G.
Peter Gouras and Ralph D. Gunkel.
6. Repression of Glutaminase I in the Rat Retina by Administration of Sodium-1-Glutamate.
Jerome K. Freedman and Albert M. Potts.
7. A Correlation of Histochemical and Electron Microscope Studies on the Rat Monkey Retina.
Arnall Patz, Arlene R. Seaman and Joseph W. Berkow.
8. The Metabolism of Retinal Pigment Cell Epithelium: The *in vitro* Incorporation of P^{32} and the Effect of Diaminodiphenoxyltane.
Vora Glocklin and Albert M. Potts.

MONDAY

2:00 P.M.

9. The effect of pupil Size on Accommodation, Convergence and the AC/A Ratio.
Harris Ripps, Newton B. Chin, Irwin M. Siegel and Goodwin M. Breinin.
10. Loss of Ocular Motility in Humans During Positive Acceleration.
T. D. Duane, E. L. Beckman and K. R. Coburn.
11. The Transient Relationship Between Pressure and Volume Changes in Living and Dead Rabbit Eyes.
J. Eisenlohr and Maurice Langham.
12. Evaluation of the Basic Validity and Clinical Usefulness of the Mackay-Marc Tonometer.
Robert A. Moses, Elwin Marg and Raymond Oeschli.
13. Alterations of the Blood Aqueous Potential in the Rabbit.
James E. Miller.
14. The Measurement of Rate of Aqueous Flow with Iodide.
Bernard Becker.
15. Transport of Amino Acids into the Intraocular Fluids. I Alpha Amino Iso Butyric Acid.
D. V. N. Reddy, V. Everett Kinsey, Beverly Skrentny and Kay Hopkins.
16. Ocular Zinc Concentrations.
Miles A. Galin, Hugo Nano and Theodore Hall.

TUESDAY, JUNE 27

2:00 P.M.

Symposium on the Cornea

WEDNESDAY, JUNE 28

2:00 P.M.

Symposium on the Cornea

MIDWINTER NATIONAL MEETING

Detroit, Michigan

December 4, 5 and 6, 1961

Abstracts of papers to be presented must be submitted to the Secretary by August 15, 1961.

Write for abstract forms to Monte G. Holland, M.D., Secretary.

Chairman of Local Arrangements Committee is:

A. D. Ruedemann, Jr., M.D., 1633 David Whitney Bldg., Detroit 26, Michigan

SECTION MEETINGS

MID SOUTHERN SECTION

Oklahoma City, Oklahoma

November 10 and 11, 1961

Deadlines for abstracts—October 1, 1961

Chairman Ray Balyeat, M.D., 554 Pasteur Bldg., Oklahoma City, Oklahoma

Secretary Samuel B. Johnson, M.D., 611 Medical Arts Bldg., Jackson, Mississippi

EAST CENTRAL SECTION

Pittsburgh, Pennsylvania

January 7, 1962

Deadline for abstracts—November 1, 1961

Chairman Murray McCaslin, M.D., 550 Grant St., Pittsburgh 19, Pennsylvania

Secretary V. Everett Kinsey, M.D., 690 Mullet St., Detroit, Michigan

WESTERN SECTION

Monterey, California

November 10-11, 1961

Deadline for abstracts—October 1, 1961.

Chairman Milton Flocks, Medical Plaza, Palo Alto, California

Secretary Daniel G. Vaughan, M.D., U. of Calif. Medical Center, San Francisco 22, California

EASTERN SECTION

Bethesda, Maryland

February 17-18, 1962

Deadline for abstracts—January 21, 1962

Chairman Ludwig von Sallmann, Ophth. Branch, Nat'l Institutes of Health, Bethesda 14, Maryland

Secretary Theodore W. Sery, M.D., Wills Eye Hospital, Philadelphia 30, Pennsylvania

MIDWESTERN SECTION

Kansas City, Kansas

April 29-30, 1961

Deadline for abstracts—Time of presentation

Secretary T. F. Schlaegal, Jr., M.D., Indiana Univ. Medical Center, Indianapolis, Indiana

ANNOUNCEMENT

To members of the Association for Research in Ophthalmology:

It is with considerable pleasure that the Board of Trustees can now announce that the Association will start publishing its own Journal to be called *Investigative Ophthalmology* on a bimonthly basis starting January 1, 1962. The Mosby Company of St. Louis has been selected as publishers.

All full members of the Association for Research in Ophthalmology will receive a free subscription to the Journal. Subscription price to others will be \$12 per year.

Dr. Bernard Becker has kindly agreed to accept the Editorship of the new Journal. He will be assisted by an Editorial Board consisting of the following:

Dr. James Allen	Dr. Jin Kinoshita	Dr. Albert Potts
Dr. Elmer Ballintine	Dr. V. Everett Kinsey	Dr. George Smelser
Dr. John Harris	Dr. Irving Leopold	Dr. Kenneth Swan
Dr. Michael Hogan	Dr. Kenneth Ogle	Dr. Lorenz Zimmerman

To assure continuity in publication policy and at the same time to divide the work load and responsibility, the tenure of the members of the Editorial Board will be limited to from one to three years.

The policies of the Association with regard to publications, for the present at least, will correspond closely to those adhered to during the past years when papers presented at Association meetings were published as supplements to the *American Journal of Ophthalmology*.

The deadline each year for abstracts of papers to be presented at the June meeting is January 15 and for the December meeting, August 1. They are to be sent directly to Dr. Frank Newell, Chairman of the Program Committee. They should be informative rather than merely descriptive of the research performed. If the papers are accepted for the program, the author will be notified promptly and he must then send an original and one carbon copy of the completed manuscript to Dr. Bernard Becker by April 15 for the June meeting and October 1 for the December meeting.

All papers presented at National meetings will continue to be the property of the Association and is accepted by the Editorial Board will be published in *Investigative Ophthalmology*. Authors are invited to submit papers presented at Sectional meetings to the new Journal but this is not obligatory. Other manuscripts dealing with ophthalmic research originating either in this country or abroad may likewise be submitted for publication.

An early issue of *Investigative Ophthalmology* will contain a detailed description of instructions to authors and probably further details about editorial policies.

The Trustees feel that they would be amiss if they did not acknowledge at this time the great contribution to the Association which Dr. Derrick Vail and associate editors of the *American Journal of Ophthalmology* have made by publishing the proceedings of the Association for so many years. We therefore take this opportunity to thank Dr. Derrick Vail, Mrs. Katherine Chalkley, and those other members of the staff of the *American Journal of Ophthalmology* who have unstintingly given of their time and efforts in behalf of the Association for Research in Ophthalmology.

The next National Meeting of the Association will be held June 26-28 at the Plaza Hotel in New York in conjunction with the Sectional meeting of the A.M.A. Monday all day will be devoted to free papers and Tuesday and Wednesday afternoons to a Symposium on the cornea.

We hope you will be able to attend.

Sincerely,

V. Everett Kinsey, Chairman

Association for Research in Ophthalmology

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Address original papers, other scientific communications including correspondence, also books for review to Dr. Derrick Vail, 700 North Michigan Avenue, Chicago 11, Illinois; Society Proceedings to Mrs. Katherine F. Chalkley, Lake Geneva, Wisconsin. Manuscripts should be original copies, typed in double space, with wide margins.

Exchange copies of the medical journals should be sent to Dr. F. Herbert Haessler, 561 North 15th Street, Milwaukee 3, Wisconsin.

Subscriptions, application for single copies, notices of changes of address, and communications with reference to advertising should be addressed to the Manager of Subscriptions and Advertising, 664 North Michigan Avenue, Chicago 11, Illinois. Copy of advertisements must be sent to the manager by the 10th of the month preceding its appearance.

Change of address notice should be received not later than the 10th of the month prior to the issue for which the change is to go into effect. Both old and new addresses should be given.

Author's proofs should be corrected and returned within forty-eight hours to the Manuscript Editor, Mrs. Katherine F. Chalkley, Lake Geneva, Wisconsin. Fifty reprints of each article will be supplied to the author without charge. Additional reprints may be obtained from the printer, George Banta Company, Inc., Curtis Reed Plaza, Menasha, Wisconsin, if ordered at the time proofs are returned. But reprints to contain colored plates must be ordered when the article is accepted.





